FUNCTIONAL DURABILITY OF A QUARTZ CRYSTAL MICROBALANCE SENSOR FOR THE RAPID DETECTION OF *SALMONELLA* IN LIQUIDS FROM POULTRY PACKAGING

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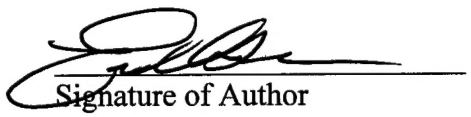

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Eric Vincent Olsen, son of Alford David and Noella Teresa (Rose) Olsen, was born January 19, 1963 in Juneau, Alaska. He graduated from Brandon High School in Brandon, Florida in 1981 and entered military service for six years. In 1989, he completed an Associate of Science in Chemistry at Galveston College. Working as a Medical Technologist at the University of Texas Medical Branch in Galveston, Texas, he completed his Bachelors of Science in Medical Technology in 1993 with highest honors. He was subsequently inducted into The Alpha Eta Honor Society for Healthcare Professionals and listed in Who's Who Among Students in American Universities and Colleges for his scholastic achievements. In 1995, he earned a commission in the United States Air Force as a Biomedical Science Corps Laboratory Officer and currently holds the rank of Captain. He graduated from the University of Southern Mississippi with a Master of Science in Education in 1997, and in 1998 he was awarded a prestigious Air Force Institute of Technology Scholarship to attend Auburn University to complete a Master of Science in Microbiology. Recently, he was inducted into Sigma Xi. He is married to Brandi Marie, daughter of Michael and Connie Myatt, and has two children, Erika Paige, age 7, and Dillon Michael, age 1.

THESIS ABSTRACT

FUNCTIONAL DURABILITY OF A QUARTZ CRYSTAL MICROBALANCE SENSOR FOR THE RAPID DETECTION OF *SALMONELLA* IN LIQUIDS FROM POULTRY PACKAGING

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Foodborne disease causes an estimated 4 to 76 million cases of food poisoning with 5,000 associated deaths annually in the United States at a cost of $6.5 –34.9 billion. In poultry products, *Salmonella* is the most common pathogenic bacterial contaminant, and is responsible for the highest percentage of foodborne disease transmission. Currently, microbial detection performed by the food industry is inadequate; tainted food products routinely are sold and consumed before contamination is revealed. A rapid, sensitive (350 ± 150 cells ml^{-1}) quartz crystal microbalance biosensor, layered with heat-treated

anti-*Salmonella*-phospholipid monolayers by the Langmuir-Blodgett technique, has been evaluated by immersion testing in chicken exudate to determine the feasibility of inclusion into processed poultry packaging. Biosensor degradation and bacterial interaction were examined under darkfield microscopy and scanning electron microscopy. Results indicate that the biosensor is specific ($r \geq 0.98$), and sensitive (≥ 10 mV decade^{-1}) for up to 32-40 hours post-immersion at 4°C with an assay time of less than 7 minutes. The working longevity of the biosensor was not increased through filter-sterilization of chicken exudate. Heat-treated antibodies (56°C for 30 minutes) were required to prepare a properly functioning sensor. Observations of bacterial interaction with the monolayer by scanning electron microscopy and darkfield microscopy agreed well with the functional duration of the biosensor. The results of the evaluation suggest the potential feasibility of using the biosensor in processed poultry packaging as a preemptive tool for the rapid, specific, and sensitive detection of *Salmonella*.

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INTRODUCTION

Poultry is one of the most widely consumed meat products throughout the world today (83). Among the reasons for consistently increased annual consumption rates are its ready availability, recognition as a relatively inexpensive and healthy alternative protein source, storability, by-product variety, and availability as a "fast-food" (75, 83). Unfortunately, popularity among consumers has also lead to a substantially increased number of foodborne disease outbreaks, with microbial contamination due most notably to poor processing quality control (54).

Annually, according to the Centers for Disease Control and Prevention (CDC), the United States has approximately 40,000 foodborne illness cases reported with 1000 associated deaths (19, 22). The costs associated with food poisoning due to lost productivity, treatment, and legal expenses are enormous, ranging in the tens of millions of dollars each year (16). The primary bacterial agent responsible for gastro-intestinal disorders in the United States is *Salmonella*; with roughly 35% of these cases attributed to *Salmonella typhimurium* (41).

The detection and enumeration of microbial contamination in meat products has long been a subject of research by scientists (30). New, innovative efforts based on conventional techniques for the detection of *Salmonella* in poultry food products include PCR assay (46), optical methods (97), immunofluorescent microscopy (24), ELISA (35, 101), and improved culturing techniques using indirect conductance measurements (11).

101), and improved culturing techniques using indirect conductance measurements (11). However, these methods are limited in their application due to several factors: they require extensive amounts of time to perform, are cumbersome, are expensive, increased manpower, and do not lend themselves well to automation (36). Due to the fact that poultry is a perishable item which must be processed rapidly, assay detection times are limit and assays are not expected to determine bacterial contamination before the consumer purchases it (103). A rapid and specific and cost-effective method for the detection of *Salmonella,* that is dedicated to each poultry product at the time of processing would prevent millions from incurring sickness or death.

The *Salmonella* detection device described by Pathirana et al. (66), is well suited for the immediate detection of pathogenic bacteria in processed poultry. The biosensor is rapid, requiring less than 5 minutes assay time, and combines the specificity of antibodies with the high sensitivity of a quartz crystal microbalance (QCM). Changes in resonant frequency of the QCM due to mass and viscoelectric effects as bacteria attach to the sensor surface are used to identify the presence of bacterial contamination. These attributes make it capable of measuring specific, minute mass changes that result from bacteria binding to the antibody coated surface. Inclusion into poultry packaging during processing could allow continuous monitoring from processing to point of sale, and subsequent disposal of any contaminated poultry prior to human consumption and ensuing foodborne illness. As well, liability for contamination could be assigned which will lead to improvements in processing through the identification of points that allow contamination or growth of microbes.

The ability of the biosensor to operate for extended periods of time within poultry packaging is essential to continuous monitoring; the operational environment would entail deployment under refrigerated temperatures and immersed conditions from the time of packaging to the marketplace. Therefore, the objectives of this research and specific aims to accomplish these objectives are as following:

(i) Determine the functional longevity of the biosensor after immersion in raw chicken exudate:

a) master the construction of the biosensor using a patent-pending methodology, and operation of the microbalance to assay the biosensor;

b) define the acceptable level of the biosensor performance after immersion in order to have a minimal confidence level of correct functioning of the biosensor;

c) determine the performance of the biosensor after immersion in chicken exudate for various durations and temperatures.

(ii) Determine the biosensor specificity (the ability of the sensor to discriminate between different antigens).

(iii) Investigate methods of increasing the functional longevity of the biosensor:

a) examine effects of the filter sterilization of the chicken exudate on the monolayer degradation;

b) examine sensor reusability by reversing bacterial binding;

c) examine properties of the immunoreactant monolayer deposited from different *Salmonella* antibody preparations;

d) determine features of the monolayer degradation by electron and darkfield microscopy.

LITERATURE REVIEW

Salmonellosis and poultry processing.

Poultry is one of the most popular and important sources of food and protein throughout the world today (83). Consumption rates, particularly in the United States, are consistently rising. Currently, Americans consume 33 kg per capita annually (83, 88). Unfortunately, with increased popularity and consumption have come increases in foodborne illnesses, and in particular, salmonellosis (20, 88, 100).

Food poisoning is a pathological condition resulting from the consumption of food products contaminated with pathogenic agents such as bacteria, viruses, parasites, or toxins (54, 89). Bryan (14) states that more than 200 known diseases can be transmitted through food products. The most common pathogenic bacterial contaminant of poultry, with the highest percentage of food-borne transmission in the United States, is *Salmonella* (57, 73, 79, 85). This has been confirmed by the World Health Organization (WHO), and the Centers for Disease Control and Prevention (CDC) (21, 99,100). Sofos (85) reported that 40% of all microbial contamination in poultry could be attributed to *Salmonella* spp., while Schutzke et al. (79), estimated *Salmonella* contamination at 50%. Other common food-borne bacterial contaminants include *Campylobacter* spp., *Escherichia coli*, *Yersinia enterocolitica*, *Shigella* spp., *Staphylococcus* spp., and *Clostridium perfringens* (53, 54, 102).

Salmonella is one of 24 genera of the family Enterobacteriaceae, the largest, most heterogeneous collection of medically important gram-negative bacilli (62). The genus has grown to include over 2500 serotypes since Daniel E. Salmon, an American veterinary surgeon, discovered the first strain in 1885 (100). Of these, *Salmonella* serovar typhimurium and *Salmonella* serovar enteritidis are the most common serotypes responsible for human disease in the United States (Fig. 1) (20, 79, 100). The epidemic strain of *Salmonella typhimurium* Definitive Type 104 has been identified as possibly the worst emerging offender of the *Salmonella typhimurium* strains (4, 41, 100). When isolated from humans, *Salmonella* is almost always pathologically associated. *S. typhimurium* is estimated to cause 25-35% of all reported *Salmonella* poultry food-borne infections (89).

Salmonella typhimurium is a facultative anaerobe measuring roughly 0.3-1.0 × 2.0-6.0 μm, and is usually motile with peritrichous flagella (42). It is a chemoorganotroph, it does not form spores and can be isolated from a variety of foods, especially eggs, dairy products and poultry (54, 79, 97, 102). It is found in virtually all animals as an inhabitant of the gastrointestinal system, and spread as a contaminant via the fecal to oral route (54, 88). Ingestion of the organism from contaminated foods is the source of most infections, requiring a large inoculum (10^6-10^8 cells) for the development of symptomatic disease (54, 62, 66).

Numerous virulence factors are associated with *S. typhimurium* including endotoxin and enterotoxin production, capsule formation, adhesion factor expression, antimicrobial resistance, intracellular survival and multiplication, and sequestration of growth factors (62). The enterotoxin is heat-labile and almost identical to cholera toxin.

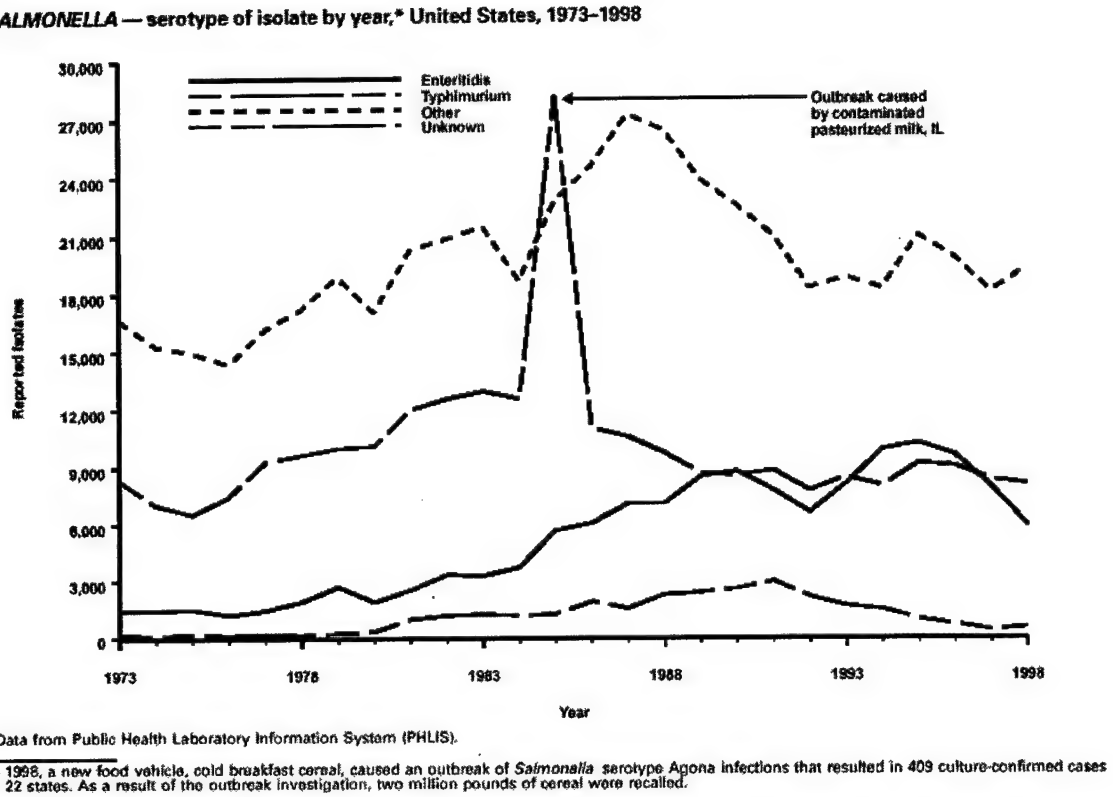

FIG 1. According to the Centers for Disease Control and Prevention, *Salmonella typhimurium* and *Salmonella enteritidis* account for 44% of the laboratory isolates responsible for human foodborne disease in the United States (19).

The expression of this toxin leads to increased cyclic-AMP levels resulting in electrolyte transport imbalance with subsequent diarrhea (62).

The most common manifestations of *Salmonella* food poisoning are the accompanying symptoms of diarrhea, fever, and abdominal cramps that develop 12 to 48 hours post-infection (22). The illness is usually self-limiting, resolving within 4 to 7 days without treatment (20). However, some individuals may require hospitalization for dehydration if the diarrhea becomes severe enough (20, 22, 89). Generally this situation is only seen in infants, elderly people, and immunocompromised individuals. In 1998, the CDC reported 43,694 cases of salmonellosis in which age groups 1-4 and 40-64 had the highest number of reported cases; 6,975 and 5,927 respectively (19). Systemic salmonellosis may also occur in these individuals through crypt cell invasion, resulting in blood and organ involvement, and ultimately death (89). Prompt treatment involves rehydration with intravenous electrolytes and systemic antibiotics such as ampicillin, gentamicin, trimethoprim /sulfamethoxazole, or ciprofloxacin (21, 89). However, an increasing number of strains of resistant *Salmonella* are being seen due to the overuse of antibiotics to promote the growth of feed animals and poultry and may prolong excretion of the offending agent (53, 89). The epidemic strain of *Salmonella typhimurium* Definitive Type 104 is resistant to at least five antimicrobial drugs (4, 100). With infection, some individuals may require several months before their bowel habits return to normal due to upsetting the normal flora of the intestine (89). A small number of infected individuals develop Reiter's syndrome, a form of chronic, reactive arthritis that may last for a prolonged period of time (21). These individuals are debilitated with joint pain, conjunctivitis, urethritis, cervicitis, and rarely cardiovascular involvement (89).

As of January 1, 1998, the CDC designated a total of 52 infectious diseases as notifiable at the national level, and tracked monthly, of which salmonellosis is one. A notifiable disease is one for which regular, frequent, and timely information regarding individual cases is considered necessary for the prevention and control of the disease (18).

It has been estimated that foodborne diseases cause approximately 76 million illnesses with 325,000 hospitalizations and 5,000 deaths in the United States alone each year (57). Buzby et al. (16), estimates 3.3-12.3 million cases annually with 3,900 associated deaths. The WHO (99) quotes Buzby et al. (16), in their quarterly statistics report, but goes on to state that morbidity and mortality may reach as high as at 6.5 – 33 million cases with 9,000 deaths. In addition, they state that foodborne illnesses may be 350 times more frequent than reported. Tamblyn et al. (88), conservatively estimates 4 million salmonellosis cases; this information is confirmed by the CDC (20). Regardless of the estimates, only 40,000 of these cases are actually reported annually to the CDC (22). Statistics show that reported cases of salmonellosis have increased in the past thirty years (Fig. 2). Mead et al. (57), states that the inconsistency between estimates and actual cases numbers is due to poor tracking. Clinicians are not required to report case numbers, and diagnoses based on laboratory results are usually not performed unless hospitalization occurs. Another study that may explain the difference is that most people do not seek medical attention due to the self-limiting nature of the disease. Therefore, the case numbers for illnesses, hospitalization, and deaths due to salmonellosis have been grossly underreported, possibly by as much as 38 fold (57). Statistics show that salmonellosis is more common in the summer, with 6,034 cases occurring in August of

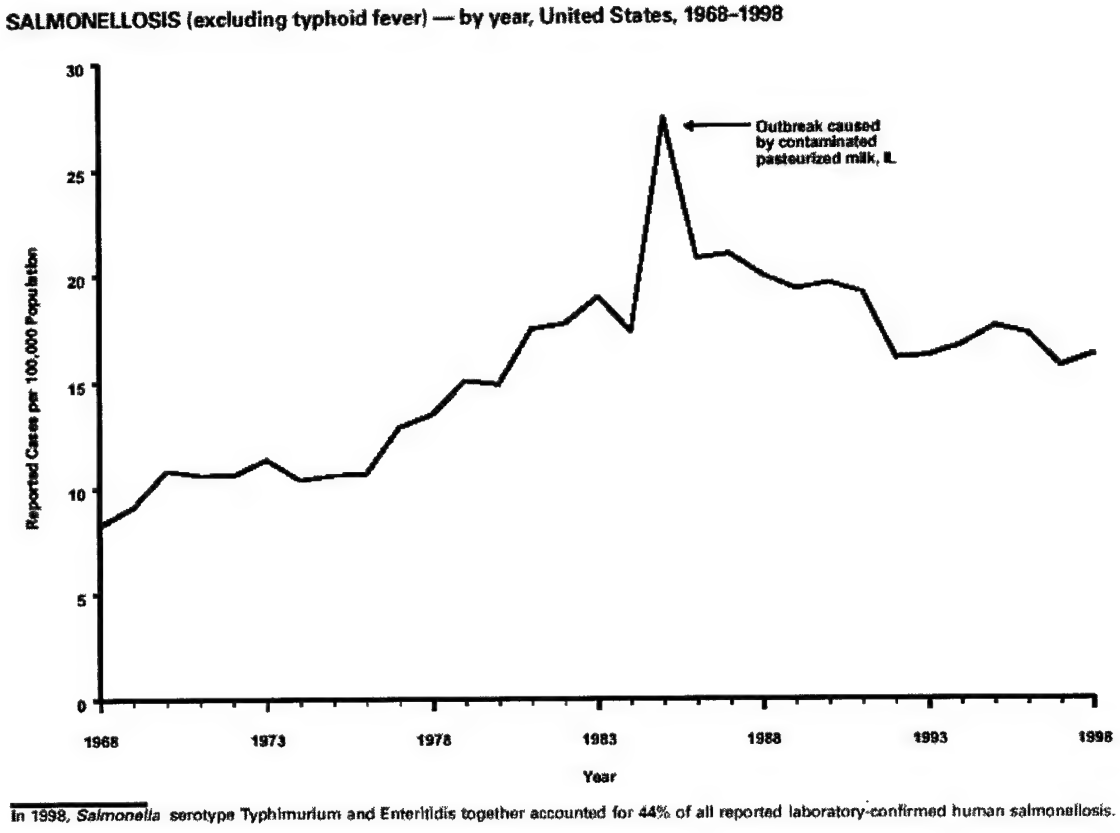

FIG 2. According to the Centers for Disease Control and Prevention, reported cases of salmonellosis have increased almost 100% over the past 30 years (19).

1999, and more cases are reported in the South Atlantic region of the United States with 9,326 cases reported in 1998 (19). This peak incidence is probably a result of increased outdoors-social gatherings in which the contaminating offenders are consumed.

The monetary cost of bacterial food poisoning can be staggering. Buzby et al. (16), estimated the costs of human illness in the United States alone to be between $6.5 - $34.9 billion annually. Another study of the United States estimated the patient-related costs of salmonellosis to be between $275 million and $6 billion per year (79). The WHO estimated the annual cost of salmonellosis in the United States to total almost $4 billion annually (100). Cost estimates per reported case of human salmonellosis range from $1,000 to $1,300 in North America and Europe (100). In 1994, the United Kingdom Food and Drink Federation calculated that foodborne illness, principally salmonellosis, was costing the British economy about £1 billion annually (100). The costs associated with individual outbreaks in North America and Europe range from around $60,000 to more than $20 million (100). These costs reflect the substantial economic drain affecting individuals, families, industries, legal systems, health care systems and entire communities. Epidemics of foodborne diseases can also affect tourism and trade. The WHO reports that when cholera broke out in Peru in 1991, over $700 million were lost in fish and fishery products exports (100). In the three months following the start of the epidemic, $70 million were lost due to closure of food service establishments and a decrease in tourism.

The substantially large economic drain and microbiological quality of processed poultry is a major concern of processing facilities (76, 97, 102). Public awareness and increasing numbers of foodborne illness cases have necessitated closer examination of

livestock cleanliness and poultry processing (45, 85, 88). Normally, healthy birds carry extensive amounts of microbes on their skin and feathers (85). During the growth of the bird, the natural defenses of the animal keep the microbial population in-check. However, when the bird is slaughtered and processed for market, the carcass becomes extremely vulnerable to a host of microorganisms. Marriot (55) estimated that roughly 1 billion microorganisms are contained in 1 gram of soil attached to the hide of a live animal; fecal material can hold as many as 220 million microbes/gram, and one square centimeter of the animal's unwashed skin holds as many as 155 million microorganisms. These organisms are easily transferred during processing when improper evisceration and poor thermal processing may cause cross-contamination between carcasses (76, 83). Silverside and Jones (83) report that although *Salmonella* does not grow quickly in the processing plant, and hardly at all below 7°C, it can continue to survive on the carcass and become a health hazard later. Slaughter of the animal can introduce the bacteria internally during the bleeding of the bird (55, 85). The carcass has not been defeathered at this point and the flora of the bird's hide can be directly introduced into the meat through the puncture wound to the throat. A major, if not the largest cause of cross contamination of microbes between healthy and contaminated birds, results during the scalding step when slaughtered birds are placed together in large vats at 50-60°C (88, 96). Scalding for roughly 45 seconds aids in the removal of the outer skin and feathers of the carcass (75). However, even at this temperature, the vats can still have a bacterial load of up to 1 billion microorganisms per liter of water (55). Another route of contamination is evisceration of the bird after slaughter and defeathering of the carcass. During this time, fecal contamination can occur if the gut is not completely empty of food; the stomach and

intestines are loaded with normal flora and notably *Salmonella* (85). To alleviate this problem, farms initiate feed withdrawal at the point where the bird has reached maturity and is ready for slaughter (75).

Marriot (54) states that the majority of bacterial contamination occurs through cross-contamination as a result of poor sanitation practiced by handlers during processing. The education of food handlers in the principles of safe food handling is an essential step towards reducing the incidence of food-borne disease resulting from cross-contamination during processing and preparation of food (100).

The chilling of the carcass during processing is extremely important in maintaining bacteria at low levels while increasing shelf life and organoleptic quality (73). If temperature regulation is not closely regulated during the chilling process, destruction of microorganisms won't be complete resulting in contamination of multiple birds bathed together (77, 83). Studies have shown that significant cross contamination occurs during water chilling, used almost exclusively in the United States, and could be readily decreased by using the air chilling process used almost exclusively in European countries (30, 75, 77, 102,).

The control of contamination in poultry seems to be best achieved through cleanliness and proper sanitation. However, bacterial contamination during processing may be unavoidable because some birds are so heavily infected (88). In these cases, rinsing during chilling may fail to remove any significant amount of *Salmonella* because the organism binds so tightly to the carcass that they become part of the food product. The contamination of one bird can cause 100% contamination of all finished products (55). Because of unavoidable contamination, a bacterial immunosensor that allows

continuous monitoring of pathogenic bacterial levels, and is capable of inclusion into the processed packaging could prevent the sale of contaminated poultry when processing methods fail.

Microbial detection using biosensors.

Currently, the meat processing industry performs microbial detection and enumeration on the processed end product (96). Processed specimens are randomly selected from the production line and analyzed most commonly through ELISA procedures, although some conductance methods have been described (12, 68). Other conventional techniques employ selective culture media, biochemical or serological characterization (70). While these methods are sensitive and specific, they may take up to several days or even weeks to determine if the product contains strains of pathogenic bacteria (7, 12, 65, 70). A more expedient alternative is polymerase chain reaction, which is utilized by the industry to a small degree (65, 67). However, it is expensive, labor intensive, limited by inhibitors present in some food products, and may detect DNA from dead cells (65, 67). During the time required to perform testing, larger processing plants may have distributed a contaminated product to points of sale before the problem is realized. Subsequently, consumers may ingest the tainted products before a recall can be performed. An example illustrating this is the case of Hudson Foods, where 25 million pounds of ground beef were recalled due to contamination by *E. coli* O157:H7 (25). United States Department of Agriculture officials stated that much of the beef had already been sold through fast food outlets and well known supermarkets, and probably consumed before the recall was announced (93). At least 20 cases of food poisoning were associated with the contamination (93). Federal records indicate that in 1999,

almost 5 million pounds of meat and meat by-products were recalled, yet less than 4.5% were recovered (94).

In 1992, the National Advisory Committee on Microbiological Criteria for Foods implemented the Hazard Analysis and Critical Control Point (HACCP) system as a rational means of insuring food safety from harvest to consumption (63). The HACCP system relies on critical control points (CCP) as maximum and minimum deviation limits. Deviation from these limits might put the consumer in danger of foodborne illness (63). The 1997 HACCP document states that microbiological testing is seldom effective due to its time-consuming nature and problems with assuring the detection of contaminants (63, 95). However, it is important in the overall program to monitor the CCP. Therefore, faster and more reliable methods of detecting foodborne pathogens would benefit the system overall (95). A rapid, accurate, and sensitive method for the detection and enumeration of microbial contaminants in food products has long been a subject of research by scientists (30). This need has led to new innovative techniques, such as biosensor assays, which will eventually replace traditional methods of food analysis such as ELISA that are slower, labor-intensive, and cost-inefficient (38, 58). Blankstein and Larsen (10) state that biosensor assays and their miniaturization can improve the reliability and speed in which analytical determinations are made, while reducing the sample size, analysis time, and assay costs.

A biosensor can be most simply defined as "the spatial unity of a physical transducer and a biological recognition system" (1). Hence, it consists of two main components; namely some sort of receptor that has affinity for the particular analyte of interest, and a transducer to convert sample coupling to a usable signal output (61). The

signal may then be amplified and processed to provide quantitative measurement of the ligand-receptor interaction.

The history of biosensors has been documented from 1962 when Clark and Lyons published their essay on a reusable enzymatic electrode (1). Since then, engineering approaches used in the development of biosensor assays have been well documented (36, 40, 61, 78); and can be categorized into optical, biochemical, electrochemical, and acoustic wave/mass change methods. The measurement of mass change is based on the acoustic wave properties of piezoelectric (PZ) crystals, which are more commonly known as quartz crystal microbalances (QCM). QCM have been the subject of intense research since the reporting of the first analytical application of a PZ crystal in 1964 (47) and the first antibody-based biosensor in 1972 (81).

The QCM consists of a thin AT- or BT-cut thickness shear quartz crystal wafered between two circular electrodes applied on both sides. This substrate can have varying dimensions and resonant frequencies. AT and BT describe resonator plates cut from the original crystal at precise angular orientations that are well known to quartz crystal unit manufacturers, and thickness shear describes the motion of the quartz plate as it oscillates (8). The piezoelectric properties and crystal structure of the quartz result in a shear deformation of the crystal when a voltage is applied across the electrodes (usually made of gold), which induces an oscillating field perpendicular to the surface of the crystal (58). This characteristic produces a standing wave of oscillation in the quartz, whose direction is dictated by the cut of the crystal. The AT-cut crystals (used predominantly) displace the oscillation parallel to the wafer's surface (58). The crystal can be excited into resonance oscillation when placed into an external oscillatory circuit with a

frequency such that the crystal thickness is an odd multiple of half of the acoustic wavelength (8). The QCM is very sensitive to the accumulation of surface mass since the frequency difference of the unloaded and loaded crystal can be measured precisely and decreases proportionally to an increase in the deposited mass on its surface.

When coated with a layer of antibody, the QCM is an excellent method of detecting and enumerating bacteria in liquid medium and has two distinct advantages: it is inexpensive and relatively simple, requiring only an oscillatory circuit and voltage or frequency counter (84). Temperature and fluid viscosity effects can be eliminated by use of a reference sensor. Frequency decreases primarily reflect mass and viscoeleastic effects caused by bacterial deposition and binding at the surface (71). QCM biosensors are similar to traditional direct immunoassay coupling systems that are highly specific and sensitive to a target organism. The biosensor receptor corresponds to a target antigen and consists of a specific antibody immobilized to the surface of the QCM (that acts as the transducer) through a reliable deposition process. The binding of the target antigen with the immobilized high affinity antibody, most commonly of an IgG class, results in a change in the transducer's oscillation properties at the sensor's surface. This oscillation shift is normally measured as a frequency change, which is lowered proportionally to the increase in the mass.

Measurement of the frequency change of the quartz crystal prior to and after the addition of the target bacteria while mounted into an oscillation unit has been described (48, 49, 58, 60, 65, 68, 69, 84). However, Ye et al. (103), state that this system is an inefficient analysis system, and describe the use of flow injection analysis (FIA) for their PZ biosensor. Chen and Karube (23) have also reported the use of FIA for the detection

of *Salmonella* in food products. Phase shift measurements, due to oscillation mode jumps in their device, have been utilized by Welsch et al. (98).

Because of their antibody-based methodology, mass biosensors are highly specific and most commonly known as affinity immunosensors (1, 61). The specificity of immunosensors has been well documented (48, 49, 60, 68, 69, 103). When combined with their rapid bacterial identification and ability to enumerate cells, they are well suited for the detection of bacteria in food products (68). Assay times have been reduced from days to hours and minutes, and sensitivities are comparable to conventional assays. Yu and Bruno (104) have documented an immunosensor with a detection limit of 1.0-2.0 × 10^3 cells ml $^{-1}$ and an assay time of less than 1 hour for the detection of *E. coli* O157:H7 and *S. typhimurium*; however, the sensor was not linear. Park and Kim (65) reported an assay time of 30–90 minutes using a thiolated *Salmonella* biosensor with a detection limit of 9.9×10^5 cells ml $^{-1}$ and detection range up to 1.8×10^8 cells ml $^{-1}$. Ye et al. (103) reported on a 25 minute linear response biosensor assay ($r = 0.942$) for *S. typhimurium* with a detection limit of 5.3×10^5 cfu ml $^{-1}$ and a range up to 1.2×10^9 cfu ml $^{-1}$. Minunni et al. (58) designed a linear response QCM sensor ($r = 0.99$) for *Listeria monocytogenes* with a detection time of 15 minutes. The *E. coli* biosensor of Pyun et al. (69) had a detection range of 3.0×10^5 to 6.2×10^7 cells ml $^{-1}$. The *Salmonella* biosensor of Prusak-Sochaczewski and Luong (68) was rapid with an assay of 50–60 seconds and a lower detection limit of 10^5 cells ml $^{-1}$. However, it required a 0.5 - 5 hour incubation time depending on the concentration of the microbial suspension. Foremost, however, is the rapid and sensitive biosensor designed by Pathirana et al. (66), for *Salmonella*.

Capable of assay times of 79 ± 20 seconds, the sensor requires no incubation period, has a sensitivity of 18 ± 5 mV decade^{-1} of *Salmonella* concentration, and a linear ($r = >0.98$, $p < 00.1$) detection range of 350 ± 150 cells to 10^{10} cells ml^{-1}.

The construction of the immunosensor first requires preparation of the substrate surface for acceptance of the antibody. The electrodes of the QCM sensor must be extremely clean. Generally, this involves thorough cleaning of the surface using NaOH and/or HCl or another type of acid, followed by rinsing with distilled water to remove all contaminants that may affect the deposition process. The next step is immobilization of the antibody to the surface of the transducer-substrate. Prusak-Sochaczewski et al. (68, 69) and Luong and Guilbault (52) have described several methods, including the application of thin polymer or silane layers, biotin-avidin interaction, and glutaraldehyde cross-linking. Other methods documented include antibody thiolation (65), polyethylenimine-glutaraldehyde (PEG) and dithiobis-succinimidyl propionate (DSP) coupling (39, 48, 86, 103), Protein A-Gold (48, 49, 98), Protein G (58), enzymatic immobilization (76), alternating monolayers of monoclonal antibody against anti-horse radish peroxidase and dextran sulfate (15), and Langmuir-Blodgett (LB) (66).

Park and Kim have called for the development of new antibody immobilization methods in order to produce sensitive and reliable biosensors (65). The Langmuir-Blodgett method described by Pathirana et al. (66), is a unique advance towards antibody immobilization onto a solid platform. When combined with antibodies, the LB film is a biological membrane that organizes the antibody proteins into active assemblies that retain their biological activity and special recognition properties in the form of alternating

self-organized monolayers (2, 6). These soluble antibodies have a F_c (fraction-crystallized) portion that is hydrophobic, anchored into the lipid monolayer. As a portion of the film, the antibody will be positioned in the correct orientation with the Fab (fraction-antibody) portion oriented outward to capture the target antigen. Heat-treating the antibodies at high temperature (usually 56°C for 30 minutes) is normally performed to effect hydrophobic conformational changes to the Fab portion, opening it up to increase its ability to bind the target antigen (44). Ahluwalia et al. (3), state that the LB method produces a high density coating of antibody, but has high non-specific binding and poor reproducibility. However, by using a patent-pending LB technique, good specificity for *Salmonella* has been achieved by Pathirana et al. (66), but with a fabrication yield of only 40%. Barrud et al. (6), have stated that the LB method is quite suitable for biosensor preparation; playing a large part in the field of bioanalytical chemistry now, and continuing to do so in the foreseeable future. They go on to state that the LB method is uncomplicated and an excellent tool for building well-engineered monolayers with controllable characteristics, and when applied to PZ crystals, produces a QCM that is an extremely accurate detector of mass change. Ahluwalia et al. (2), have documented the thermodynamics of LB films and have found them to be quite sensitive to a wide range of antigen concentrations, with the minimum sensitivity limited by the accompanying detection system used to monitor the QCM. Bykov (17) concurs that LB technology is well suited for producing nanotechnology biosensors.

Although relatively cheap to produce in mass, cost effectiveness is an issue that will need to be addressed. Recycling of the biosensor by removing the bacteria while keeping the antibody intact and functional, is most desirable. Reusability relates directly

to the stability of the antibodies on the surface of the immunosensor and the ability to reverse binding so as not to destroy the functionality of the antibodies. The literature reports very little research in this area. König and Grätzel (48, 49) have reported viral immunosensors which are highly reusable up to 18 times by surface regeneration using 0.2M glycine hydrochlorine, 0.2M ethanolamine, 8M urea, and synthetic peptide competition displacement. Prusak-Sochaczewski and Luong (68) confirmed the use of 8M urea for bacterial removal, however, the sensitivity of their sensor declined rapidly after 6-8 washings. One method that has not been noted in the literature is reversal of binding through oscillation at a resonance frequency that is higher in energy than the one used to oscillate the crystal for bacterial measurement. It is thought that a higher amount of energy will disrupt the electronic interaction of the antigen-antibody reaction sites and thus reverse the binding at the sensor surface (64).

Immunosensors have been particularly significant in the rapid detection of bacterial food pathogens such *as E. coli* (27, 70), *Listeria monocytogenes* (58), and most importantly, *Salmonella* (13, 28, 38, 51, 65, 66, 68, 69, 80, 82, 87, 103, 104, 105). The first QCM assay for *Salmonella* was described by Prusak-Sochaczewski and Luong (68). The use of QCM biosensors in microbial food contamination has been thoroughly reviewed by Luong and Guilbault (52). The ability of QCMs to function in liquid environments conducive to bacterial growth is one reason for its significance as a rapid detection tool. However, immersion testing has been restricted to that of assay time only. The ability of a QCM to function for extended periods under constant immersion without affecting the functional abilities of the sensor or the immobilized antibody is not noted in the literature. There is a need for this information as confirmed by Minunni et al. (59),

and Prusak-Sochaczewski and Luong (68) in order to produce biosensors capable of semicontinuous and continuous operation in food products.

A rapid and specific, cost-effective method for the detection of *Salmonella* which is dedicated to each poultry product at the time of processing could prevent millions from incurring sickness or death. Inclusion into poultry packaging during processing would enable continuous monitoring of all products from processing to point of sale, and subsequent disposal of any contaminated poultry prior to human consumption and ensuing foodborne illness. If contamination has occurred, liability could be assigned; possibly leading to improvements in processing through identification of offenders.

The ability of the biosensor to operate for extended periods of time within poultry packaging is essential to continuous monitoring; the operational environment would entail deployment under refrigerated temperatures and immersed conditions from the time of processing to the point of sale. The biosensor could be used as a stand-alone detection system or possibly combined with other devices such as a processor chip that could act as a mini computer (10). This would allow enhancement of the biosensor abilities, to include continuous tracking of the food products, temperature and/or bacterial concentration of the monitored organism.

Microscopic characterization of Langmuir-Blodgett films.

Microscopic characterization of LB films in general have been documented most recently through the use of scanning force microscopy (37), atomic force microscopy (72), and scanning tunneling microscopy and spectroscopy (17). The micrographs of Grunder et al. (37), revealed that surfactant films are not homogeneous and undergo phase transitions depending on the surface pressures. Rinia et al. (72) discovered that

defects in the forms of holes were present in all bilayers and the described bilayer blistering is due to elevation of the bilayers by repelling negative phospholipid charges. The recent visualization and characterization of LB antibody films has been significantly less documented in the literature. The literature includes the use of fluorescence microscopy (29, 90), and surface plasmon spectroscopy and microscopy (29).

Ulman (92) has thoroughly reviewed microscopy methods for the observation of LB films to include scanning electron microscopy (SEM), transmission electron microscopy (TEM), and fluorescent microscopy. He notes that although these methods are powerful for the visualization of organic surfaces. Many reports lack useful information about the monolayer itself and they detail the physical properties of interest. Ulman (92) goes on to state that detailed information of surface morphology is vital to the development of monolayer-based technology and he suggests that SEM and TEM should be used to detail new methods and devices involving LB films.

METHODS AND MATERIALS

Salmonella biosensor and checkslide construction

(i) Apparatus. High quality, insoluble *Salmonella* antibody monolayers for biosensors and glass checkslides were fabricated with a KSV 2200 Langmuir-Blodgett (LB) film balance system (KSV-Chemicals, Helsinki, Finland) (Fig. 3), which consisted of the following components:

Monolayer trough. Transverse-suspended, rectangular Teflon trough of dimensions 450 mm × 150 mm ×14 mm used to prepare monolayers. The trough has smooth non-adhesive surfaces, holds approximately 1.5 liters of subphase solution, and incorporates a dipping well of cylindrical dimensions 80 mm diameter × 97 mm deep at one end to allow the transference of monolayers to substrates. The trough is mounted atop a stone table of 100mm thickness in order to minimize extraneous vibrations.

Surface compression barrier. A rectangular Teflon block of dimensions 210 mm × 35 mm × 16 mm is attached to a solid stainless steel support, and interlocked to a transverse, motorized, steel fork-retainer during compression. The hydrophobic barrier makes an exacting fit with the horizontal edges of the trough to prevent film leakage during compression and maintenance of the monolayer at lower or higher surface pressures.

Surface balance Wilhelmy plate. The platinum Wilhelmy-type is a surface balance plate of dimensions 19.6 mm × 10.0 mm, and measures surface pressures in the

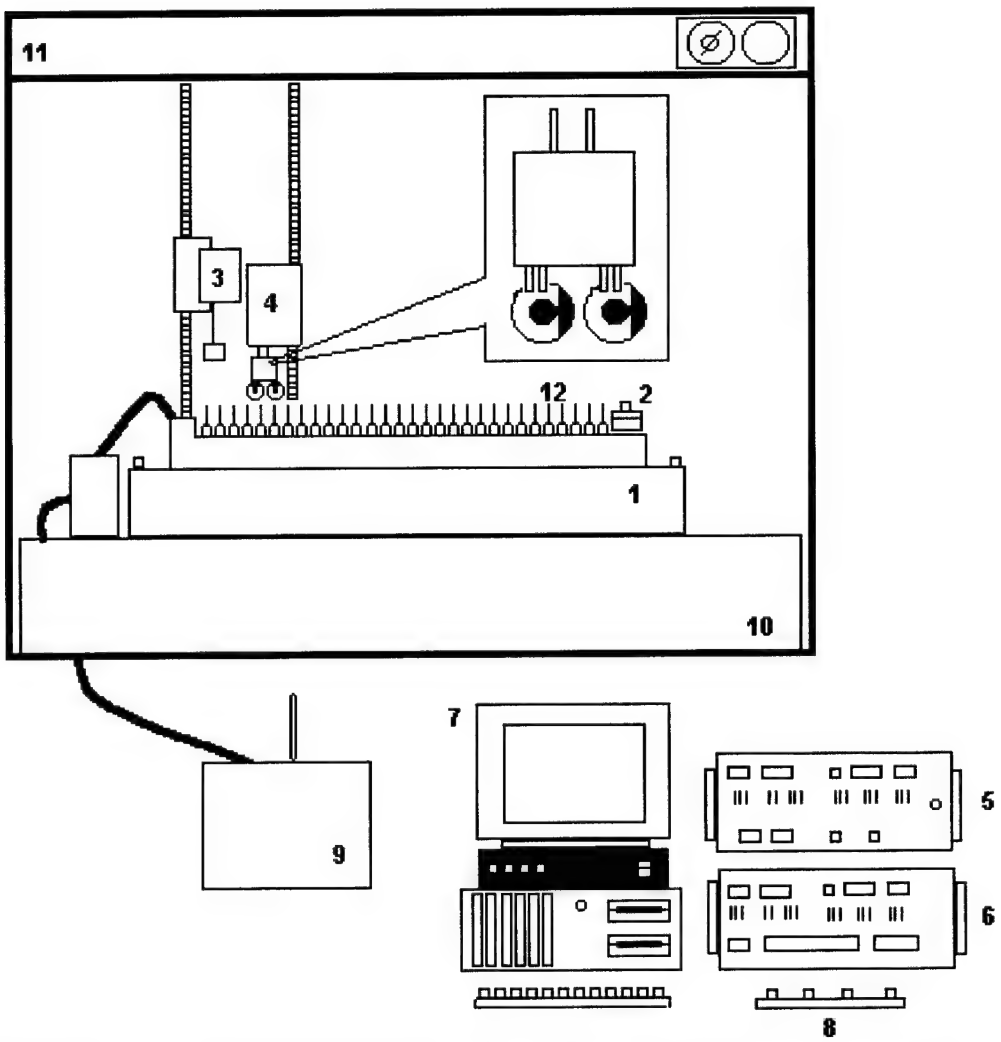

FIG. 3. Schematic of KSV 2200 Langmuir-Blodgett film balance system. 1. Monolayer trough; 2. Compression barrier; 3. Balance head with Wilhelmy-type surface balance plate; 4. Film collector unit with holder and crystals attached; 5. AFC unit; 6. DFC unit; 7. Data processing unit; 8. External control keyboard for film control mechanics; 9. Water bath; 10. Stone table; 11. Enclosure; 12. *Salmonella* antibody nolayer on surface of subphase. Inset: close up view showing orientation of crystals in holder.

balance plate of dimensions 19.6 mm × 10.0 mm, and measures surface pressures in the range of 0-100 mN m^{-1} during compression with a sensitivity of 10 μN m^{-1}. The Wilhelmy plate motorized balance head allows for precise vertical positioning into the subphase, and is interfaced to the Dynamic Film Control (DFC) surface electrobalance unit.

Film collector unit. The film collector unit is a perpendicular aligned, motorized unit that allows for exact initial placement and movement of the substrate through the compressed monolayer in order to deposit antibody monolayers onto the surface of the substrate. The automated movement of the unit is controlled through film control mechanical motors and pre-specified parameters set on the Automatic Film Collection (AFC) unit.

Film control mechanics. Mechanical management of the monolayer is controlled through stepped motor driven mechanics that direct the horizontal movements of the compression barrier, and the course vertical positioning of the surface balance and film collector unit. The motors are controlled through the AFC unit or an external control keyboard.

Automatic film collection (AFC) unit. This unit sets the functional and mechanical parameters of the substrate to include the initial positioning of the substrate above or below the subphase, the rate, distance, and direction of the vertical movement of the substrate during coating, the number of layers applied, and the delay rate of deposition between successive layers.

Dynamic film control (DFC) unit. This surface barostat unit also sets functional and mechanical parameters which include resetting of the surface pressure after initial

placement of the Wilhelmy plate, the upper and lower limits of surface pressure for isotherm determinations, automatic measurement and control of the constant surface pressure, and the horizontal rate of compression-relaxation by the barrier during monolayer depositions and isotherm determinations.

Constant temperature circulator. This device is a reservoir-type water bath (Fisher Scientific 900, Pittsburgh, PA) with a refrigerated circulator unit that maintains the subphase at a constant temperature by convection cooling. Circulating water from the bath is channeled through 13mm rubber tubing at a constant temperature to a quartz coil which circumferences the bottom of the trough. A thermocouple with an accuracy of 0.5°C allows for monitoring of the subphase temperature within the trough by liquid crystal display (50).

Data processing unit. An IBM AT compatible personal computer (Sperry 3157-00) with monochrome monitor (Roland DG MA-121A) and keyboard is used for data processing. It allows for data acquisition from the AFC and DFC and post-data analysis and processing of films and isotherms. The computer is interfaced to a asynchronous code activated switch box (Black Box Corp., Pittsburgh, PA, CAS-4Q) that communicates with both the AFC and DFC units through RS232 ports.

Trough enclosure. A laminar flow hood which surrounds the trough and maintains dust free conditions through positive pressure ventilation and a Modular Absolute Scanned filter capable of filtering 99.997% of contaminants (Dioctyl Phthalate test method, particle size 0.3μm) from the air intake (50). The enclosure also provides an illumination source; flowmeter and 5-speed flow control switch, and is isolated from the

trough and stone table on a vibration-free rubber cushion to eliminate extraneous vibrations.

(ii) *Salmonella* antisera. Three types of *Salmonella* antibody, from two different sources, were utilized in the production of biosensors and checkslides.

Commercial (Denka Seiken Co., LTD., Tokyo, Japan). Rabbit polyvalent somatic-O *Salmonella* antiserum in a phospholipid base. The concentration was not available through the manufacturer. The antibodies were heat-treated by the manufacturer at 56°C for 30 minutes, absorbed to remove cross-agglutinins, and sterilized by antibacterial filtration (74). The antiserum was stored in a brown glass vial with stopper at 4°C.

Non-commercial. This rabbit polyclonal *Salmonella* antibody was heat-treated and non heat-treated as described by Hsieh (44) (Department of Nutrition and Food Science, Auburn University, AL). Female rabbits, New Zealand White, 8 weeks of age, were immunized with 1% formalin-fixed *Salmonella typhimurium* cells ($1x10^6$ cells ml^{-1}) suspended in complete Freund's adjuvant (total 2.0 ml), and inoculated subcutaneously at multiple sites along the back. Subsequent boost inoculation (up to 5 five times) with intact *S. typhimurium* ($1x10^7$ cells ml^{-1}) suspended in incomplete Freund's adjuvant was performed every 4 weeks after initial immunization. Ten days post-boost injection, blood samples (no more than 1% of the rabbit's body weight) were collected via the central ear artery to check for specific antibody production. Bleeding procedures were repeated at 2 to 4 week intervals, with antibody titers determined each time using an indirect enzyme linked immunosorbent assay (ELISA). After titer peak, terminal blood samples were collected by exsanguination under anesthesia. Antisera was then adsorbed with non-

Salmonella organisms to eliminate crossreactive components and either heat-treated at 56°C for 30 minutes, or left untreated by heat.

The reactivity of all antiserum was evaluated for proper performance by slide agglutination tests prior to biosensor or checkslide preparation. The functional performance of the commercial antiserum was evaluated as per manufacturer's instructions (74). In principle, an antigen-antibody reaction occurs between a bacterium and its corresponding antibody to cause an agglutination reaction that can be viewed on the slide macroscopically. One drop of *S. typhimurium* suspension (1.0-2.0 x 10^{10} cells ml^{-1}) was added to one drop of the antiserum and mixed to a homogenous suspension with a sterile, wooden applicator stick. As a negative control measure, one drop of PBS was added to one drop of the antiserum on a separate slide and mixed to a homogenous suspension with a sterile, wooden applicator stick. Both slides were then rocked in circular motion for 3 minutes duration. A strong agglutination reaction was observed between the *S. typhimurium* and the antiserum after one minute of rocking, while the PBS did not react with the antiserum. The functional performance of the non-commercial antisera was assayed as described previously using the commercial antiserum reactions as a reference standard; identical reactions, both positive and negative, were observed.

(iii) Subphase solution. This liquid is a pH balanced electrolytic solution that acts as a subphase onto which the antibody preparations are spread in order to form monolayers. Impurities, in the range of parts-per-million that can collect at the surface as a result of dirty glassware or contaminants can result in poor monolayers and deposition (32). Therefore, all glassware used to prepare the subphase and stock solutions was used solely for its preparation and rinsed with only deionized double distilled water (DDH_2O) prior

to and after usage. Chemicals (Fisher Scientific, Fair Lawn, NJ) used to prepare the stock molar solutions were of the utmost purity available.

The liquid subphase was prepared in a 4 liter beaker by adding stock solutions of 20 ml of 0.02M $CaCl_2$, 20 ml of 0.2 M $MgCl_2$, 200 ml of 1.1M KCl, 20 ml of 1M NaCl, and 80 ml of 0.1M 3-(N-morpholino)-propanesulfonic acid (MOPS) to 3.5 liters DDH_2O. The pH 7.4 ± 0.1 was adjusted with 0.1M KOH and the volume brought to 4.0 liters with DDH_2O. The subphase solution was then filtered three times under gravity and stored at room temperature in an inert glass bottle tightly capped to prevent evaporation. Subphase was used within one week of preparation or discarded. Final composition of the subphase was 55 mM KCl, 4 mM NaCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM MOPS at pH 7.4 ± 0.1.

(iv) Substrates.

Biosensors. The sensors were unpolished quartz crystal sensors (Maxtek Inc., SC-501-2) used for monolayer deposition and bacterial binding measurements. These sensors are AT-cut quartz crystals of 2.5 cm diameter and 0.028-0.033 cm thickness, with a 5 MHz nominal oscillating frequency (56). The sensors have both obverse and reverse gold electrodes, over a special titanium adhesion layer, that provide an electrical connection via index pins to an external oscillatory circuit in the housing portion of the sensor probe (Fig. 4). Maxtek pre-tests the crystals to assure conformance to critical specifications that insure accuracy in rate and thickness measurements (56).

According to the manufacturer, the sensor crystals are reusable after stripping (56). In order to remove any extraneous material and the antibody film off the surface of the biosensor, recycled quartz crystal sensors were immersed in a 50% (vol/vol) solution

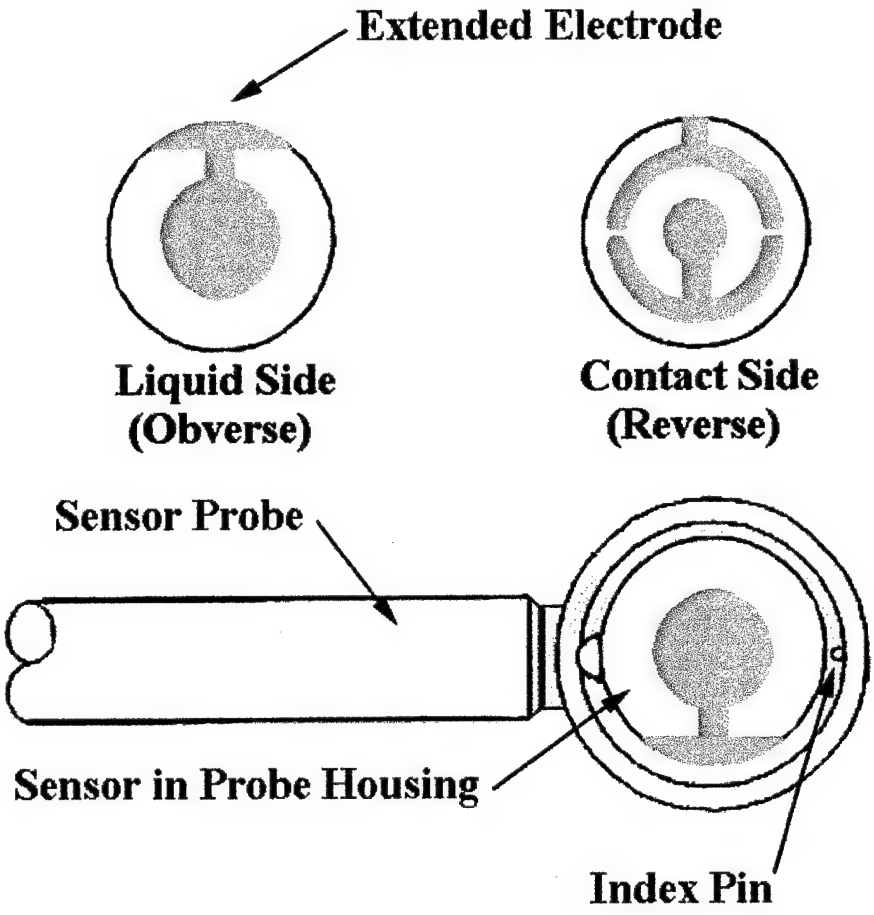

FIG. 4. Orientation of the biosensor in the probe housing of the sensor probe with the liquid side up and the reverse side contacting the index pins. The sensor retainer and hub ring are not shown.

of concentrated HNO_3 for three days prior to, and after use. Sensors were not used in experiments if any loss of performance or deterioration of the gold electrode was noticed. The crystals were then thoroughly rinsed by immersion in 5 liters of DDH_2O for two days duration. During the immersion, the DDH_2O was drained and replenished several times to allow a complete removal of HNO_3 from the sensor surface. The crystals were then air-dried and stored atop plastic o-rings in sterile petri dishes at room temperature pending new antibody monolayer deposition.

Checkslides. To enable visual observations of bacterial binding, glass microscope slides were also used as a substrate for antibody monolayer deposition. When covered with the same monolayers as the quartz crystals and subjected to the same bacterial suspensions, the glass slides (named checkslides) allowed for visualization of bacteria on the sensor surface using a darkfield microscope. The glass slides were prepared for antibody deposition with the same procedures as described previously for sensor crystals.

(v) Procedures. Immobilization of *Salmonella* antibody monolayers onto quartz crystal and glass slide substrates followed the Langmuir-Blodgett (LB) protocol as described by Gaines (32). To reduce contamination of the monolayer, the doors of the enclosure through which the trough was accessed were kept closed during the compression and deposition of monolayers.

The individual components of the instrument were powered on as applicable, and the LB film software program was started on the data processing unit. The thermostat was set at the subphase temperature of $20 \pm 0.1°C$. The trough and compression barrier were thoroughly cleaned with hexane or absolute ethanol using sterile gauze and forceps. (Extreme cleanliness is of the utmost importance since impurities in the range of parts-

per-million at the surface can result in reduction of monolayer quality and transfer ratio.) The trough was filled with 1.5 liters DDH_2O, allowing a positive meniscus of ~3 mm above the horizontal edges of the trough to be formed. Grasping the stainless steel portion of the compression barrier so as to avoid contamination of the Teflon portion, the surface was swept in a smooth horizontal left-to-right motion to remove any contaminants from the face of the water or Teflon surfaces and edges. The overspill that collected in the gutter around the outside perimeter of the trough was suctioned out using a rubber tube attached to a suction pump and house vacuum. This cleaning step was repeated twice. After the final sweeping, all DDH_2O in the trough was thoroughly suctioned out. The trough was filled with 1.5 liters of the subphase solution and again cleaned 3 times by sweeping with the compression barrier and suctioning as described previously. A ~1 mm positive meniscus was left onto which the *Salmonella* antibody would be spread. The compression barrier was securely fitted into the transverse-retaining fork.

The Wilhelmy plate, and a glass rod used to transfer the antibody to the subphase surface were thoroughly cleaned by sonication in a successive series of the following solvents for no less than 2 minutes each: 1) 2:1 chloroform/ methanol mixture; 2) acetone; 3) DDH_2O; and 4) ethanol. This cleaning procedure was performed for the initial use of the Wilhelmy plate each day. Between successive runs occurring on the same day, the Wilhelmy plate and glass rod were sonicated for 2 minutes in DDH_2O only. Using forceps, the Wilhelmy plate was suspended above the subphase from a hang-down wire arm of the electronic microbalance. The Wilhelmy plate was lowered into the subphase using the film control mechanics manipulated through the keypad; then raised

and lowered several times within the subphase to ensure complete wetting of the surface. Finally, the Wilhelmy plate was positioned within the subphase at a zero contact angle with 1/3 of its length immersed in the subphase.

The parameters of the compression and deposition of the particular film were entered into the LB instrument programs, called DFC and AFC. In the LB film program, *Salmonella* antibody monolayers were given an unknown concentration and the molecular weight was estimated to be 100,000. The volume of antibody used to prepare monolayers was variable depending upon the substrate and the type of antibody used. To fill a trough with antibody monolayer for preparation of biosensors and checkslides, 150 µl or 250 µl of commercial *Salmonella* antiserum was used; respectively. The type of subphase (*Salmonella* antibody), its pH (7.4 ± 0.1), and temperature as displayed by the LCD readout were recorded. The parameter of deposition, called perimeter of the substrate, was recorded as 104 mm. On the DFC unit, the constant surface pressure was set to 24 mN m^{-1}. The initial compression rate was set to 30 mm min^{-1}. The surface pressure was reset to zero with the surface balance Wilhelmy plate in the subphase, and the position of the compression barrier was set to zero. On the AFC unit, the vertical rate of ascent and descent was set to 4.5 mm min^{-1}, and the time delay between successive addition of monolayers was set to 99 seconds for optimal drying of the substrate.

Using clean forceps, the substrate, either 4 quartz crystal sensors (obverse exposed) for biosensors or 4 glass slides for checkslides, was arranged in a back-to-back position in an aluminum holder (Fig. 1 inset) clamped with wire retainers. The holder, with substrate, was positioned in the film collector unit as shown in figure 1, and then lowered into a starting position ~3 mm above the subphase using the manual keypad film

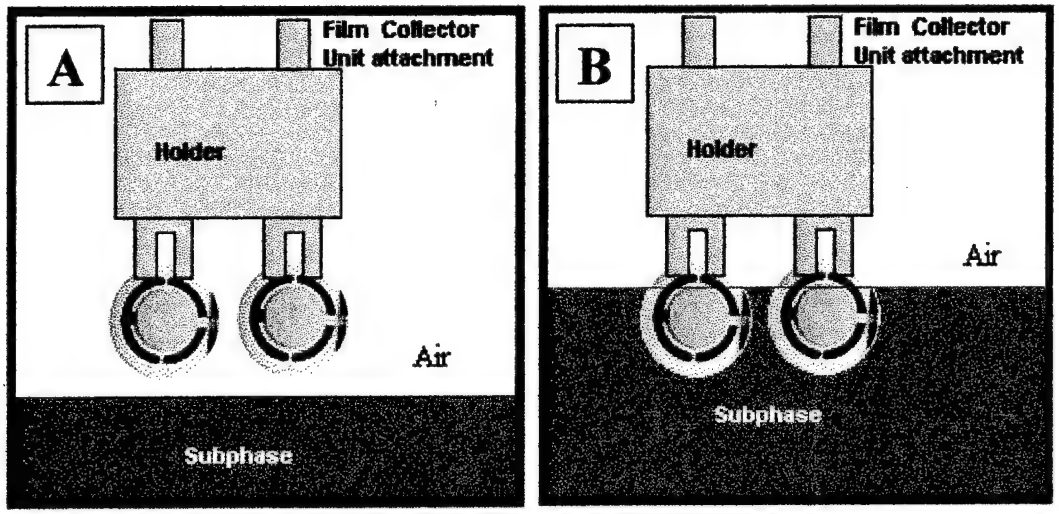

FIG. 5. Panel A: quartz crystal sensor orientation in holder. The attachment of the film collector unit is not shown. This is the starting and ending position of the sensors. Panel B: the sensors are lowered into the subphase and immersed so that the gold electrode is totally immersed but so that the holder does not touch the surface. The first monolayer is applied as the sensors rise out of the subphase.

mechanics (Fig. 5A). On the AFC, the film collector unit position was set to zero; and served as the upper limit of vertical movement for the substrate as it moves out of the subphase.

Using 150 µl or 250 µl of antibody-phospholipid, approximately 27 and 37 monolayers could be deposited onto the sensor crystal or glass slide, respectively. An odd number of layers was chosen so that when the deposition process was completed, the substrate would be left in a position above the subphase, convenient for removal of samples from the holder. The crystal was then lowered in the subphase, using the AFC film control mechanics, so that the gold electrode on the obverse side of the sensor was just below the air-liquid interface (Fig. 5B). For check slides, a lower position of 29 mm below the surface was chosen to adjust a perimeter to 104 mm. The antiserum was removed from the refrigerator, warmed to room temperature, and gently vortexed. Using an adjustable pipette with disposable plastic tip, 150 or 250 µl of antiserum, depending upon the substrate, was delivered to the subphase surface using the clean, wetted glass rod. One end of the rod was submerged below the surface while the antiserum was pipetted dropwise down the rod to the surface (Fig. 6). The drops were approximately 10 µl in volume, and it normally required 15 drops to expend all the antiserum from the pipette tip. Ten to fifteen seconds were allowed between subsequent drops to allow even distribution of the antibody-phospholipid across the subphase surface. After expending all antiserum, the tip of the glass rod was rinsed in the well portion of the trough in order to remove any excess. The monolayer was allowed to spread and equilibrate for 10 minutes, in which a surface pressure between 8-10 mN m^{-1} was normally attained.

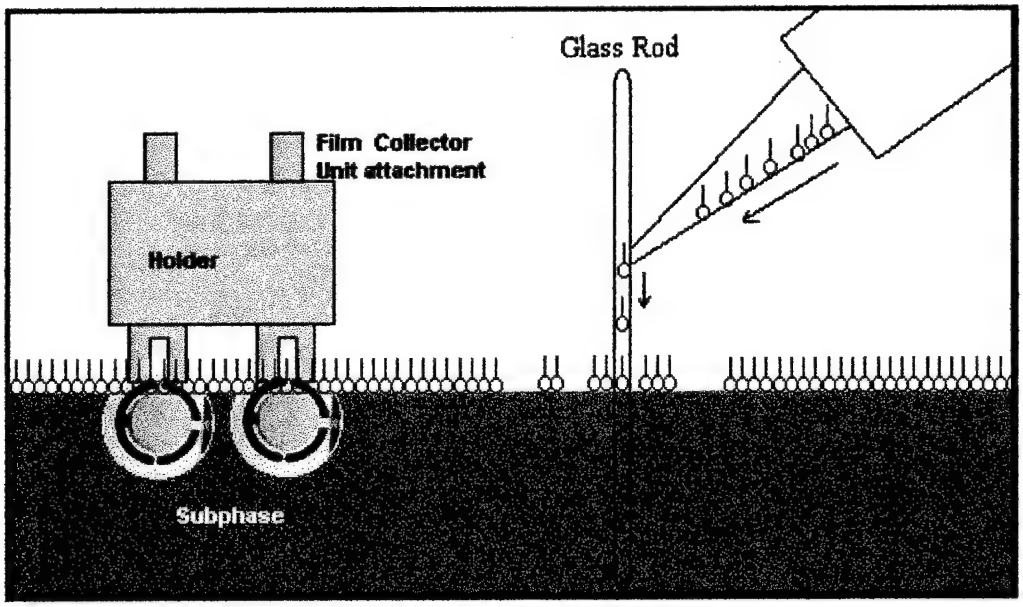

FIG. 6. Delivery of the *Salmonella* antibody-phospholipid to the surface of the subphase. The attachment of the film collector unit is not shown.

After 10 minutes, monolayer compression was initiated using the DFC. When the surface pressure reached the preset value of 24 mN m^{-1}, the compression rate was changed to 230 mm min^{-1}, and monolayer deposition to the substrate via the film collector unit, was started through the data processor and AFC unit. Acquisition and recording of deposition data, to include elapsed time, surface pressure, barrier position, and rate of deposition, was performed automatically by the data processing unit. After the final layer was deposited, the deposition process was automatically stopped by the AFC unit at the preset upper travel limit of the film collector unit, and the biosensors or checkslides were removed from the holder using forceps. The biosensors or checkslides were placed into individual petri dishes and stored at 4°C until use although Pathirana et al. (66) reported that prepared biosensors preserve their properties for up to thirty days when refrigerated at this temperature. All biosensors or checkslides were used within one week of construction.

(vi) Surface pressure isotherms. The surface pressure at which to form an organized, optimally compressed monolayer for transfer to the substrate was determined by analysis of surface pressure/surface area isotherms and elasticity of the monolayers. The procedure for the measurement of the surface pressure isotherm is very similar to one previously described for the monolayer deposition with some minor changes. The individual components of the KSV instrument were powered on and the isotherm software program started on the data processing unit. The thermostat was set at a subphase temperature of 20 ± 0.1°C. The entire trough and compression barrier was thoroughly cleaned. The trough was filled with 1.5 liters of the subphase solution and the surface swept to leave a 1 mm positive meniscus onto which the *Salmonella* antibody-

phospholipid was spread. The compression barrier was securely fitted into the transverse retainer fork. The Wilhelmy plate and the glass rod were thoroughly cleaned. The Wilhelmy plate was suspended above the subphase from a hang-down wire arm of the microbalance head using forceps. The Wilhelmy plate was lowered and totally immersed into the subphase, and left in a position so that 1/3 of its length was immersed in the subphase.

The parameters for the instrument were set in the isotherm program and DFC unit. In the program, *Salmonella* antibody monolayers were given an unknown concentration and the molecular weight estimated to be 100,000. The volume of antibody used to prepare the monolayers of the isotherm was variable depending upon which antibody was used; 1 μl was used for the commercial Denka-Seiken antiserum, 0.4 μl was used for the non-commercial Auburn University heat-treated antiserum, or 0.5 μl was used for the non-commercial Auburn University non heat-treated antiserum. The type of subphase (*Salmonella*-antibody), its pH (7.4 ± 0.1), and temperature were recorded and displayed by the LCD readout. On the DFC unit, the lower and the upper limits of surface pressure of the DFC unit were set to 0 and 80 mN m^{-1}, respectively. The constant pressure rate was set to 24 mN m^{-1} and compression rate to 30 mm min^{-1}. The starting and final areas of the monolayer were set to 0.0 and 450 mm, respectively. The surface pressure was reset to zero with the Wilhelmy plate in the subphase and the position of the compression barrier was set to zero.

The antiserum was removed from the refrigerator and gently vortexed. Using an adjustable pipette with disposable plastic tip, the respective amount of antiserum was

delivered to the subphase surface using the glass rod, which was then rinsed in the subphase. The antibody monolayer was allowed to equilibrate for 10 minutes. After 10 minutes, the compression of the monolayer was initiated at 30 mm min^{-1} through the DFC unit. Compression continued until the monolayer broke. Data collection was then terminated. A surface pressure/surface area isotherm was graphed and the monolayer elasticity was calculated.

***Salmonella* biosensor and checkslide evaluation.**

(i) Organisms. *Salmonella typhimurium* and *Escherichia coli* O157:H7 cultures from the Auburn University culture collection were used in the experiments. The identities of the isolates were confirmed through cell morphology, biochemical tests, slide agglutination, and pulsed-field gel electrophoresis.

(ii) Preparation of test solutions. The functional performance of biosensors and checkslides was evaluated using a graded series of test solutions prepared from the organisms.

One pure colony from a subculture plate was inoculated into 50ml of prepared trypticase soy broth (30g trypticase soy agar QS 1000ml with deionized distilled water, pH 7.3 ± 0.2) and incubated at 37°C in an agitating water bath for 10-12 hours. Following incubation, the broth culture was gently mixed to assure a homogenous suspension then equally apportioned into two 50ml 30mm × 115mm conical tubes (Becton Dickinson, Franklin Lakes, NJ), centrifuged (3600 × *g* for 10 minutes), and decanted. The cells were washed by resuspension in 10 ml sterile 1x phosphate buffered saline (PBS) (Molar concentrations of constituents adjusted to pH 7.4 ± 0.1) and

centrifuging at 3600 ×*g* for 10 minutes, followed by decanting of the supernatant. This step was repeated twice. After the last wash, the cells were resuspended in 7ml 1X PBS. The concentrated 7ml solution was serial diluted four times by transferring 2ml concentrate into 8ml 1X PBS throughout to make dilutions ranging from 1:5 to 1:625.

All procedures were performed in a microbiological safety cabinet, and aseptic techniques were used throughout all procedures to ensure generation of pure test suspensions. All test solutions were prepared the day of biosensor testing and maintained on ice or at 4°C until use.

(iii) Enumeration of bacterial concentration. Measurement of the bacterial cell count in the concentrated test solution suspension was carried out using an optical enumeration method. The darkfield microscopy set up described by Pathirana, et al (66), was employed (Fig. 7). It consisted of an Olympus BX50F microscope (Olympus Optical Co., LTD, Japan) equipped with a 100W high pressure Hg burner illumination source (Olympus, BH2-RFL-T3), Hg lamp (Olympus U-ULS100HG), Naessens dark-field condenser (COSE Corp., Canada), polarizer, and 100X 0.1 IRIS oil immersion objective (NA 1.35). Images from the microscope were transferred to a color video monitor (Sony Corp., Trintron PVM-1343MD) via a 0.55X CCD video camera (Diagnostic Instruments, HR055-CMT) equipped with a power supply (Optronics Engineering, DEI-470), and interfaced to a Dell Optiplex Pentium PC (GMT 5166) with monitor (Sony Corp., Trinitron Multiscan 17SFII). Images were captured and processed using 2.0 Image-Pro Plus program and 1.0 Pro Series Capture Kit (Media Cybernetics, MD). Hard copies of images were made using a video cassette recorder (Sony Corp., SVO-9500MD) or color video printer (Sony Corp., UP-3000). The microscope

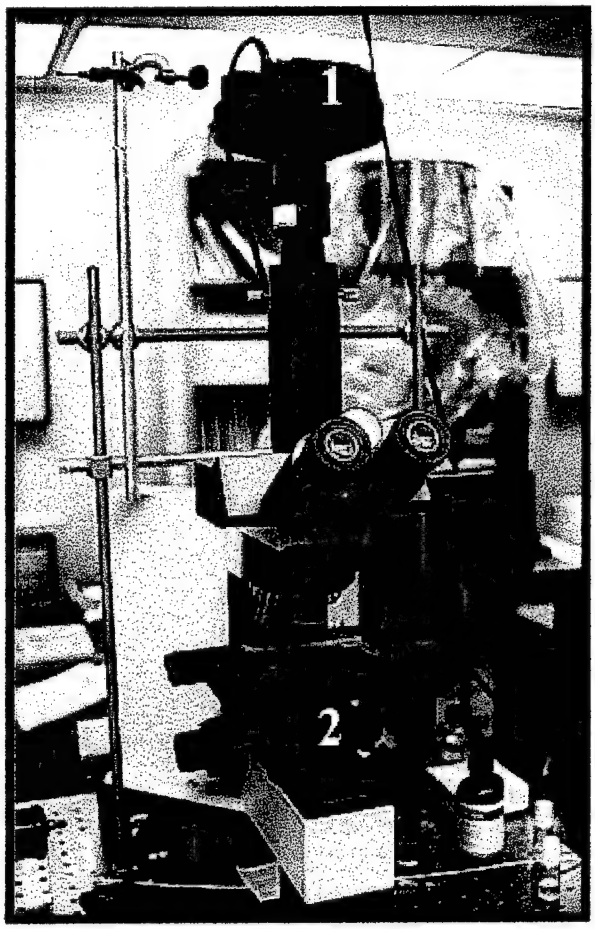

FIG. 7. Naessens darkfield microscopy setup. 1. 0.55X CCD Camera; 2. Naessens condenser; 3. Antivibration platform.

components were positioned on an antivibration platform to eliminate extraneous vibrations.

Ten microliters of the well-mixed concentrate suspension was deposited onto a clean glass slide overlaid with coverslip and examined under a 100X objective. Ten fields were captured digitally and counted using the Image-Pro Plus program. The sum of the ten different fields was divided to calculate an average number of cells per field, then divided by the volume of suspension as displayed by the video monitor to calculate the average number of cells per ml of suspension. This volume is calculated by the formula:

$$Volume\ displayed = l \times w \times h,$$

where $l \times w$ is microscope displayed area and h is the thickness of the sample. By substituting l, w, and h with the respective experimental values we have:

$$Volume\ displayed\ (V) = 72\times10^{-4}\text{cm} \times 53.3\times10^{-4}\ \text{cm} \times 10\times10^{-4}\ \text{cm} = 3.84\times10^{-8}\ \text{cm}^3$$
$$V = 3.84\times10^{-8}\ \text{ml}$$

The average number of cells ($N_{average}$) was then divided by this volume to derive an absolute bacterial count (N) for the concentrate in cells/ml given by the equation:

$$N = N_{average}/V = N_{average}/3.84\times10^{-8}\ (\text{cells/ml})$$

The bacterial cell counts of the dilutions were then calculated by multiplying the concentrate in cells/ml by the respective dilution factor of 1/5, 1/25, 1/125, or 1/625.

(iv) Apparatus. The microbalance apparatus (Fig. 8) as described by Pathirana, et al

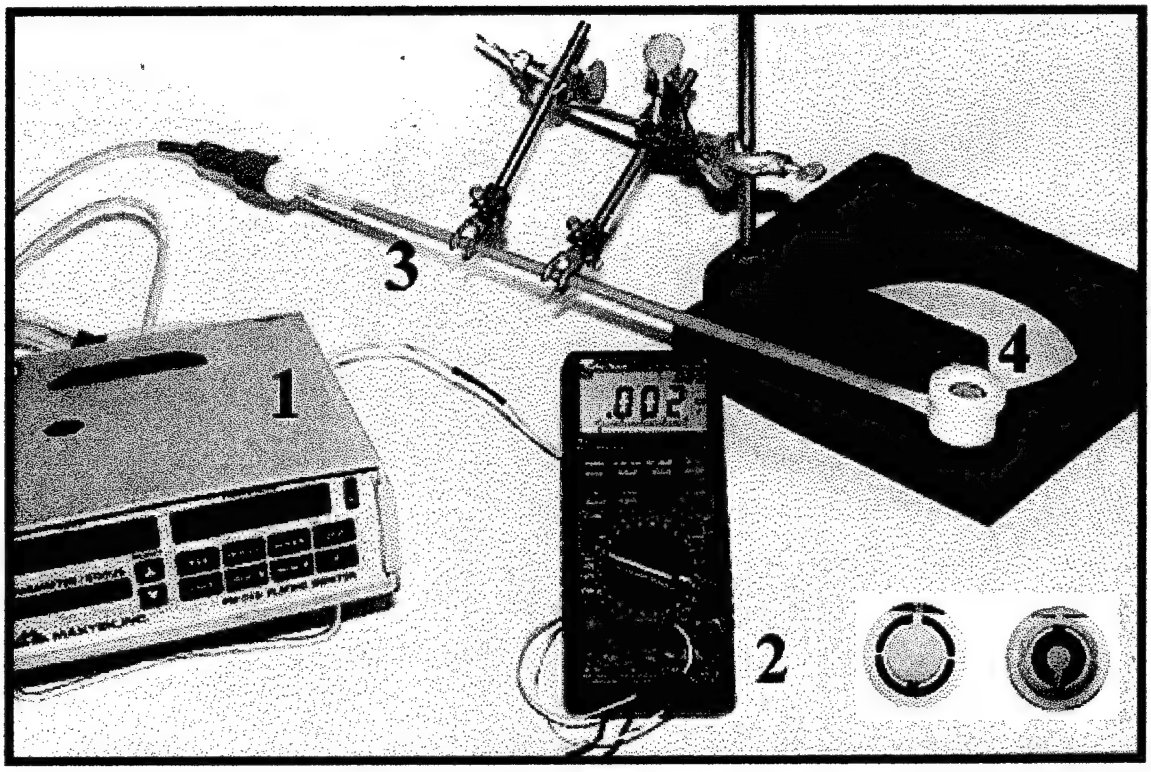

FIG. 8. Microbalance apparatus for bacterial binding measurements. 1. Plating monitor; 2. Digital multimeter; 3. Sensor probe; 4. Sensor probe housing with sensor crystal installed under the plastic retainer ring and hub. Inset: obverse and reverse of sensor crystals.

(66), was utilized and consisted of a frequency counter (Maxtek Inc., PM-740) with a frequency resolution of 0.5 Hz at 5 MHz and a mass resolution of 0.01 $\mu g/cm^2$, equipped with a 50 cm sensor probe (Maxtek, Inc., TPS-550) connected via a triaxial sensor cable (Maxtek, Inc., PC-3-0300) through a rear converter port (56). The sensor probe was positioned horizontally by attaching it to a clamp stand, positioned atop a ~1 inch thick foam pad in order to reduce vibrations. The crystal to be assayed was mounted with the reverse side down onto two index pins within the sensor probe housing which connect to the sensor oscillator electronics located below the positioned crystal, and then secured into the housing of the probe with a plastic retainer ring and screw-type hub (Fig. 9). The plating monitor was interfaced to a data processing unit (IBM PC compatible ACMA 386-25 MHz computer with Mitsubishi monitor) through a digital multimeter (Radio Shack, 22-168A). The multimeter was interfaced to the plating monitor via pins number 1 and 2 on the "Thickness" digital to analog connector of the input/output port (DAC I/O), in order to receive the frequency output of the biosensor in volts (Fig 10). The scale factor of the thickness DAC was adjusted to 5.00 microns so that the DAC voltage output was linear from 0-5 volts DC as the thickness of the accumulated bacteria increased from 0-5.00 microns. The multimeter was interfaced to the data processing unit via an RS232C cable, where the voltage responses were acquired and captured using the Scopeview software supplied with the multimeter. The battery-powered multimeter was configured to run on AC via a 9V adapter.

The voltage output from the Maxtek device is directly related to the resonance frequency of the quartz crystal sensor. Changes in the resonance frequency of the quartz crystal sensor were used to monitor the binding of bacteria to the sensor surface. The

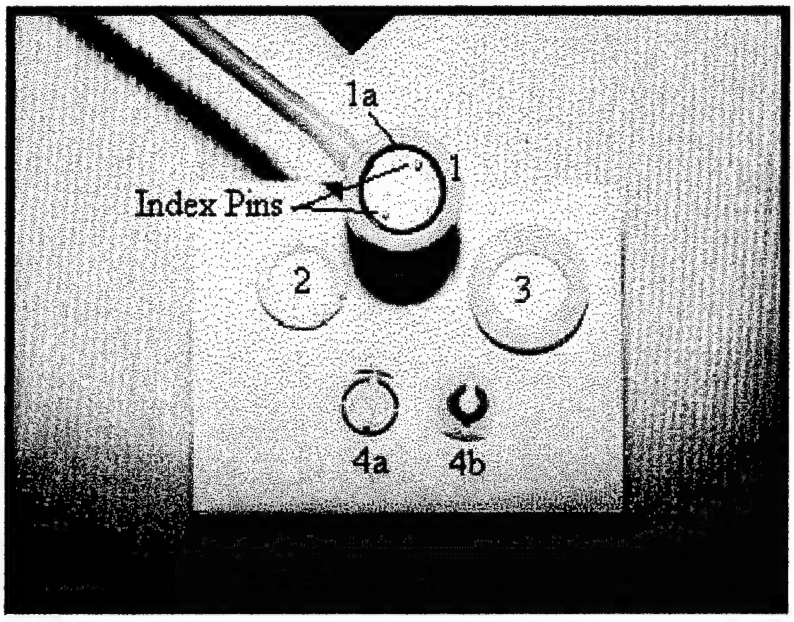

FIG. 9. Close up of sensor probe. 1. Sensor probe housing; 1a. Rubber O-ring 2. Plastic retainer ring; 3. Screw-type hub; 4a. Sensor (obverse); 4b. Sensor (reverse). The index pins are visible and contact the electrodes on the reverse side of the sensor.

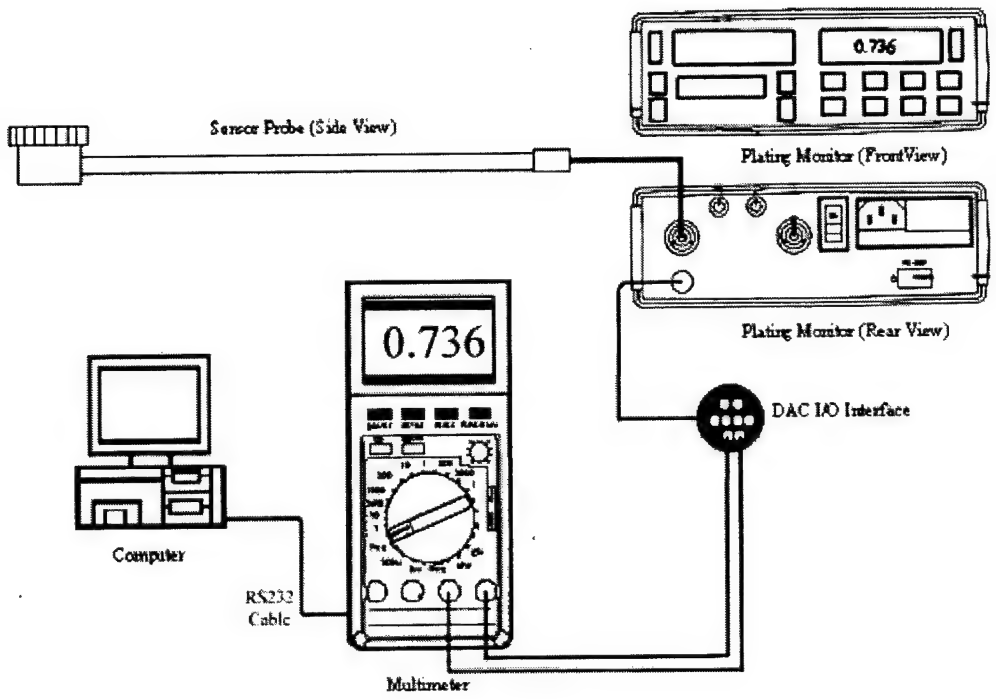

FIG. 10. Schematic of microbalance setup showing the four main components. The Thickness DAC port must be configured in order for the multimeter to read the output of the plating monitor in volts.

observed changes in the resonance frequency of the quartz crystal sensor during binding of bacteria is hypothesized to be due both to viscoelastic changes of the LB film-bacteria-near surface fluid media and the mass change associated with the binding of bacteria.

(v) Procedure. The sensor probe housing, rubber o-ring, plastic retainer ring, and hub were thoroughly cleaned with ethanol and allowed to air-dry. The rubber o-ring was positioned into the groove within the housing to keep the sensor level against the index pins, and to prevent liquid from reaching the circuit electronics. The biosensor to be tested was equilibrated to room temperature if applicable (10 minutes), then placed into the sensor probe housing using forceps with the obverse side up and reverse side down contacting the index pins of oscillatory electronics circuit. The sensor was secured with the sensor retainer ring and hub 1/8 turn beyond finger tight. The plating monitor was then reset to zero the sensor voltage response. The Scopeview application in the data processing unit was started and a corresponding file was setup for the test solution that was to be assayed. The test solution series; 1:625, 1:125, 1:25, 1:5 dilutions of the concentrate, the concentrate, plus an aliquot of the subphase, were then assayed from least concentrated to most concentrated. One ml of the subphase solution was pipetted onto the top of the sensor, and data collection of the voltage response was initiated on the Scopeview program at a rate of one data point per second. The top of the probe housing was covered with a 1 inch diameter petri dish lid to prevent air currents from interacting with the liquid. After 7 minutes, data collection was stopped. The test solution was gently suctioned off the sensor with the pipettor without touching the gold electrode of the biosensor. This procedure was then repeated in turn for each of the remaining bacterial solutions. After the last test solution was suctioned off, the sensor retainer ring

and plastic hub were removed to expose the biosensor, which was removed from the sensor probe housing using forceps. The procedure was then repeated for subsequent sensors that were tested consecutively the same day. All voltage response files were maintained in the database of the computer or copied to floppy disk and analyzed off line. After testing, the sensors were immediately immersed into ethanol for disinfection until they could be transferred to a 50% (vol/vol) solution of concentrated HNO_3 in order to strip any extraneous material and the antibody film off the surface of the biosensor.

(vi) Microscopic observation. The darkfield microscopy setup (Fig. 7) was also employed for the optical confirmation of bacterial attachment to checkslides that were immersed in conjunction with biosensors during longevity experiments. As previously described, anti-*Salmonella* monolayers were deposited onto glass microscope slides by the LB method. Whereas, the quartz crystal substrate of the biosensors is unpolished and opaque, the transparent checkslides allowed for direct microscopic observations. Using this indirect method, a comparative understanding of what may occur with the antibody monolayer of the biosensor during long-term immersion could be attained. After thorough air drying, the checkslide was secured to the stage of the darkfield microscope, flooded with 10µl of *S. typhimurium* concentrate from the biosensor testing solutions, and overlaid with coverslip. The slide was observed under oil immersion for any agglutination reactions. Observations were captured using videocassette recording (VCR). Quality control (QC) checkslides, identical to normal checkslides but unimmersed, were also tested in this same manner with *S. typhimurium* concentrate and observed in order to QC the checkslide production process. These reactions were also recorded onto VCR.

Functional duration testing

(i) Chicken exudate. Three hundred to four hundred milliliters of raw chicken exudate from processed poultry was used to immerse biosensors for longevity experiments. Whole chickens or chicken parts in sealed plastic bags under refrigerated conditions were purchased from a local supermarket. In a microbiological safety cabinet, the excess exudate was aseptically drained from the bag and chicken, and strained from the underlying absorbent pad into a sealable, autoclaved plastic container and stored at 4°C until further use.

(ii) Biosensor and checkslide retention in chicken exudate. In order to allow maximum exposure of the antibody monolayers to the chicken exudate, a slotted-Teflon block capable of retaining up to 40 biosensors in a vertical position was employed (Fig. 11). Prior to use, the Teflon block was autoclaved to ensure sterility. The block was then fully immersed to the bottom of the plastic container in preparation for longevity testing.

(iii) Procedure. Longevity testing was conducted to investigate the ability of the sensors to function at an acceptable level after immersion in raw chicken exudate under differing conditions of time and temperature. For each experiment, 28 biosensors and 7 checkslides were prepared by the LB method no more than seven days prior to test. Using sterilized forceps, the biosensors and checkslides were positioned in the Teflon block immersed in the chicken exudate within the plastic container. The lid was secured, and the container was then placed within a refrigerator (Sanyo, SR4991X) or incubator (Fisher Scientific) depending upon the experimental conditions (Fig. 12). Four separate longevity experiments were conducted at temperatures of ~ 4°C, 11°C, 23°C and 33°C. To further investigate the time dependence of functional duration within a 48 hour span

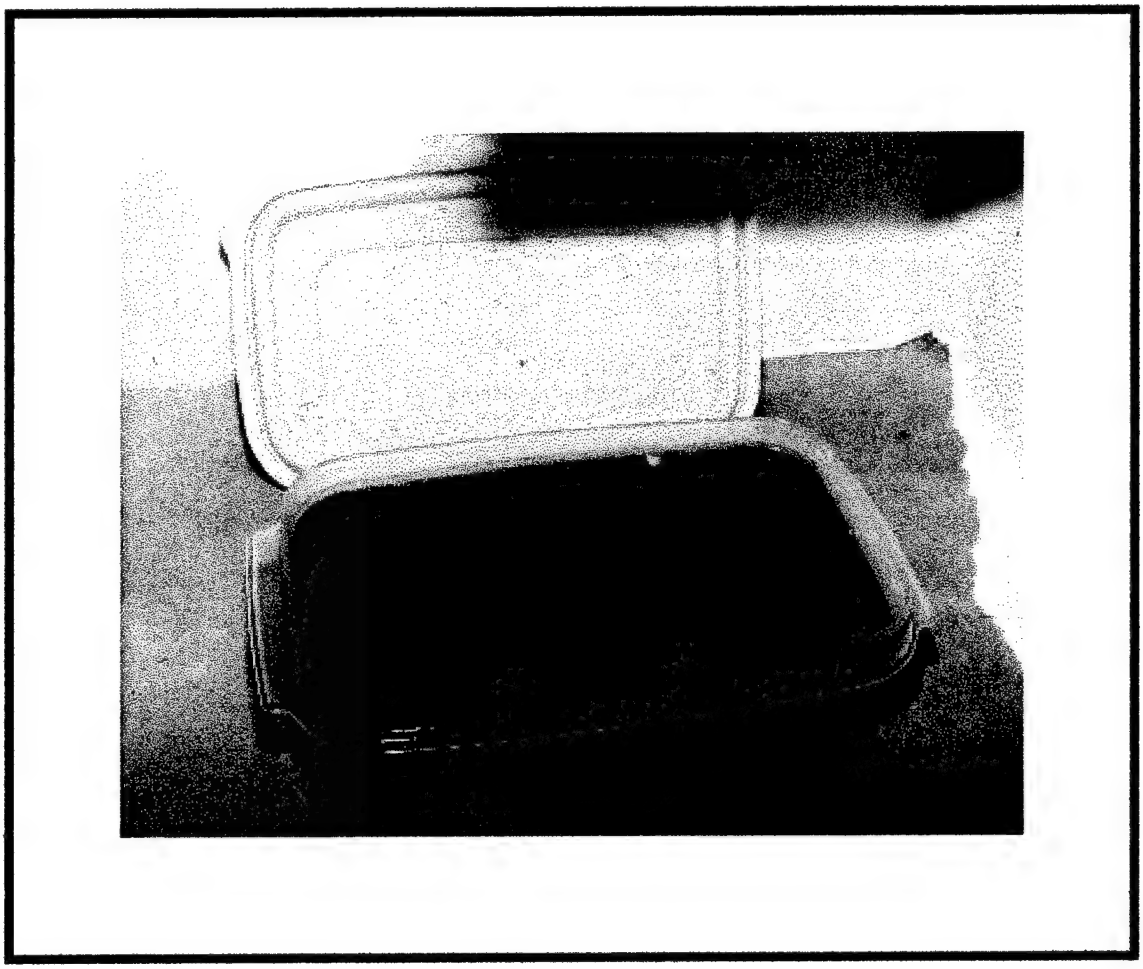

FIG. 11. Biosensors (arrow) immersed in chicken exudate under refrigerated conditions during longevity experimentation. The sensors were originally positioned vertically in the slots of the immersed Teflon block to keep them separated, but were removed and laid horizontal for this picture. During the experiment, the plastic container was sealed after placing the sensors to prevent extraneous contamination.

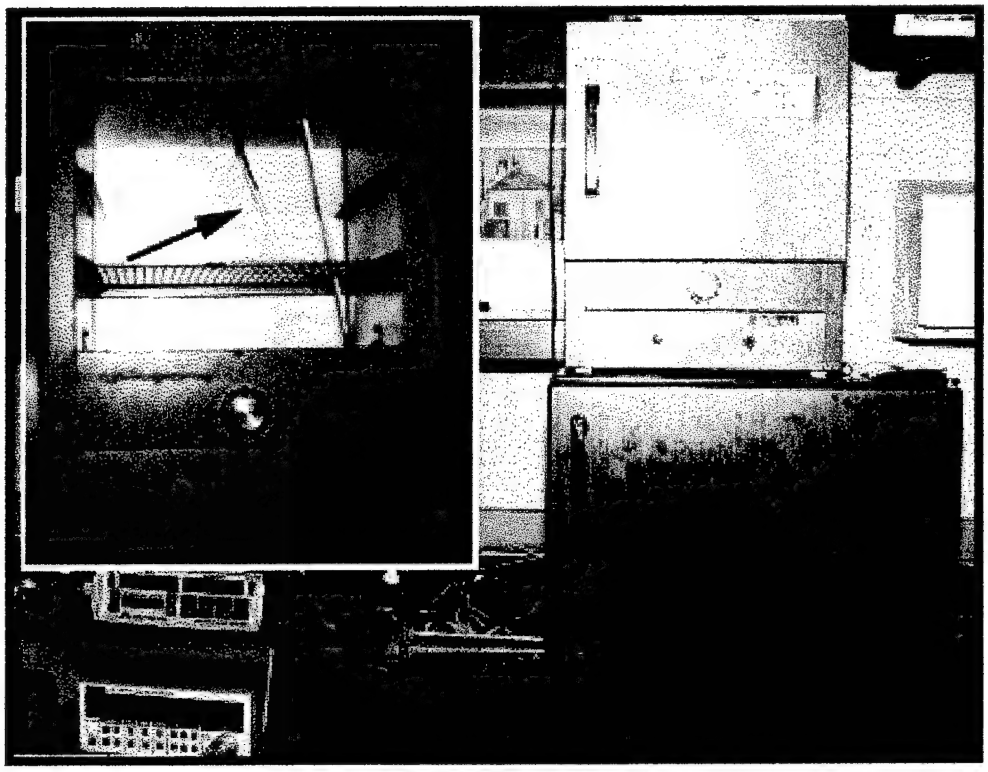

FIG. 12. Incubator and refrigeration setup for longevity experiments as depicted by the schematic in figure 13. Inset: Inside of incubator shows placement of thermistor (arrow).

of time, an additional experiment at 4°C was conducted after these experiments. An incubator was used for the experiment at 33°C.

For each experiment, each day for 7 days, 4 biosensors and 1 checkslide were removed from the chicken exudate, rinsed by immersion in subphase for up to 1 minuteto remove any extraneous material, and allowed to air dry. The biosensors and checkslides were then tested by the procedures previously described. Checkslides prepared for QC purposes were concurrently tested as described previously.

(iv) Environmental monitoring. The temperature within the incubator or refrigerator was continuously monitored with a thermistor (Victory Engineering Corp., Union, NJ, 31A6-1kOhm). The thermistor was placed within the refrigerator or incubator connected to a digital multimeter (Radio Shack, 22-168A) through the COM and V/Ω terminals (Fig. 13). The multimeter was in turn interfaced with an IBM PC compatible ACMA 386-25 MHz computer. The resistance readings of the thermistor were acquired and captured using the Scopeview software supplied with the multimeter. The battery-powered multimeter was configured to run on AC via a 9V adapter. Calibration of the refrigerator's thermistor was performed by immersing both the thermistor and thermometer (Eberbach Corp., Hg 40°C, ± 0.05 °C) into water. The water had been initially heated to 30°C, and then allowed to cool (using ice), while resistance and temperature readings were taken at selected intervals. The incubator's thermistor was calibrated by heating the unit with the thermistor and thermometer inside, while resistance and temperature readings were taken from room temperature to 40°C. During longevity experiments, resistance readings were recorded

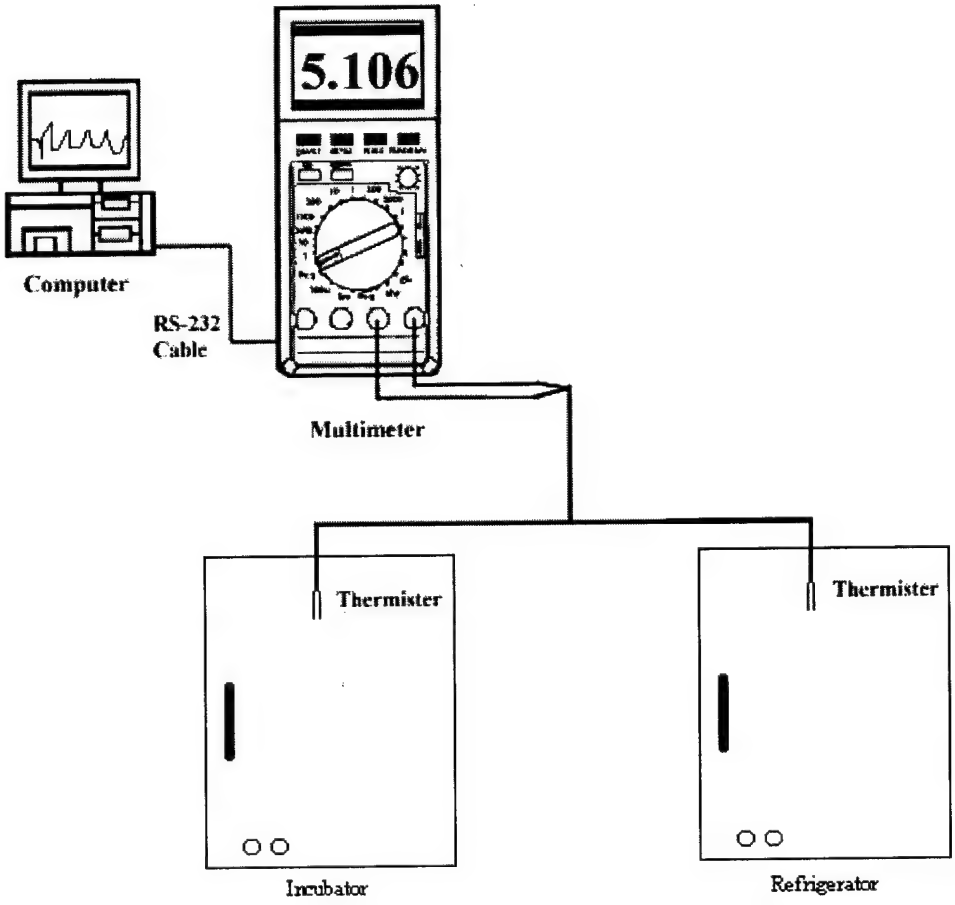

FIG. 13. Schematic of longevity experiment temperature-monitoring setup. Either the refrigerator or the incubator was hooked up depending upon the temperature of the experiment.

every minute after the placement of the container in the respective appliance. The resistance readings were then converted to temperature (°C) by plotting them against the calibration curve for the respective appliance.

Specificity testing of the biosensor. To confirm the specificity of the biosensor as previously described by Pathirana, et al (66), two different experiments were conducted using *Salmonella* biosensors assayed with test solutions prepared with either 1) the specific antigen, associated with *Salmonella*; 2) a non-specific antigen, associated with *E. coli* O157:H7; or 3) *Salmonella* pre-incubated with free *Salmonella* antibody.

(i) Preparation of test solutions. Two separate sets of bacterial test solutions with *S. typhimurium* and *E. coli* O157:H7 as the antigens were prepared as previously described. A third set of test solutions was also prepared following the same procedure, but amended as follows so that the *S. typhimurium* was blocked by incubation with free *Salmonella* antiserum in solution. After the final centrifugation step and decanting, the concentrated cells were resuspended in 7ml PBS. Four ml of the concentrate was decanted into a separate tube and combined with 2ml of the commercial *Salmonella* antiserum (Denka-Seiken Co., LTD., Tokyo). The mixture was gently resuspended by pipette and incubated for 4 hours at room temperature. Following incubation, the mixture was resuspended and serial diluted to differing logarithmic concentrations as previously described under test solution preparation.

(ii) Procedure. As previously described, 12 *Salmonella* biosensors with the commercial antiserum incorporated were prepared in a two-day period. The biosensors were then tested per procedure previously described and as follows: four sensors were tested using the *S. typhimurium* test solutions, 4 sensors were tested using the *E. coli*

O157:H7 test solutions, and 4 sensor were tested using the *S. typhimurium* preincubated with free antibody test solutions.

Biosensor function using alternate antibodies. The role that antibody preparation imparts to the performance of the biosensor was comparatively examined using the three different types of antibody previously described under biosensor and checkslide preparation. Specifically, the effects of heat-treating the antibody verses non heat-treating it, and using concentrated antibody verses diluted, were questioned.

(i) Preparation of sensors. Twenty-four *Salmonella* biosensors were prepared by the LB method using both heat-treated and non heat-treated antiserum from Auburn University as previously described over a 7 day period, with one minor change. The amount of antibody used to prepare monolayers was 25µl, as opposed to 150µl, to account for the increased concentration of the antibody.

(ii) Procedure. *S. typhimurium* test solutions were prepared as described. The 24 biosensors were tested over a two-day period as previously described.

Reversal of biosensor bacterial binding. It was suggested that the chemical bonds holding bacteria on the biosensor surface could be broken, and the bacteria can be released back in the surrounding solution if the crystal holding the bacteria is subjected to high power oscillations. To examine this possibility, oscillation at a high level of energy resonance frequency was used. A special stainless steel probe housing with no internal oscillator was built for these experiments. The probe has a sensor loading area to fit regular crystals with *Salmonella* antibody. Eight *Salmonella* biosensors using the commercial antiserum were prepared as described previously. *S. typhimurium* test solutions were prepared as described previously. Four of the biosensors were then tested

as follows. Each of these 4 sensors was first tested for binding *S. typhimurium* using the test solutions as described previously. The sensor was then washed by immersion in subphase for 1 minute, then air-dried. Then the sensor was placed into a stainless steel probe for testing. One ml of subphase solution was pipetted onto the surface of the sensor, which was then oscillated for 1 minute at 5 MHz and 200 mV with a BK Precision 4040 Sweep Function-Generator, as depicted in figure 14. The subphase was then suctioned off by pipette, and the sensor was removed, washed by immersion in subphase, and air-dried. The biosensor was again tested in the regular probe using the test solutions as described previously. Four biosensors were then tested without oscillation as described previously to act as a reference.

Functional duration testing using filter sterilized chicken exudate. Filter sterilization of indigenous microflora and biological materials from the raw chicken exudate was performed to determine the effects that filtering may have upon sensor performance; specifically, it was done to determine if filtering increased the working longevity of the sensor.

(i) Filter sterilization of chicken exudate. Two hundred milliliters of chicken exudate was collected as previously described, aliquoted into four 50 ml, 30 mm × 115 mm conical tubes (Becton Dickinson, Franklin Lakes, NJ), and centrifuged at 8000 ×*g* for 10 minutes. This step was repeated, and the supernatant from all four tubes was collected into a sterile 250ml disposable vacuum filtration system with 0.45 μm membrane (Nalge Co., Rochester, NY), and vacuum filtered. This step was repeated to net 150 ml of filtered chicken exudate. This exudate was collected into three separate sterile 50 ml

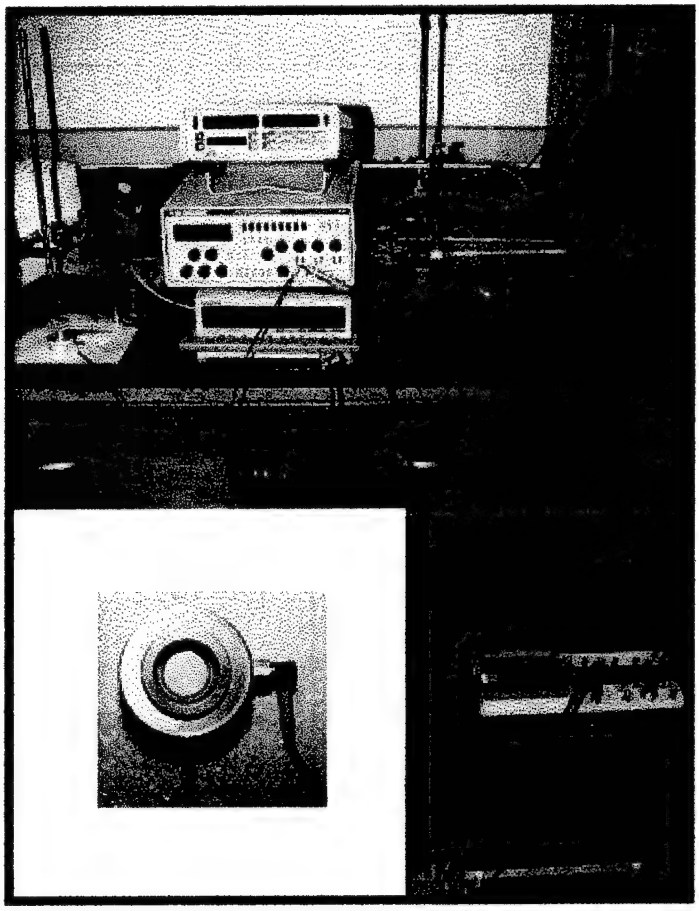

FIG. 14. Oscillation experiment setup. Sensor probe attached to oscillation unit with oscilloscope (floor). Inset: Close up of stainless steel sensor probe housing with retainer ring and hub securing an installed sensor.

disposable vacuum filtration units with 0.22 μm membranes (Millipore Steriflip, Molsheim, France) and vacuum filtered. This step was repeated to net 110 ml of clear chicken exudate. The exudate was stored as previously described in the sterile plastic container outfitted with the slotted Teflon block at 4°C until use. All filtrations, and the immersion and removal of biosensors for testing, were performed in a microbiological safety cabinet using aseptic techniques. One loop of the filtered chicken exudate was cultured on a TSA plate at 37°C to confirm proper sterility.

(ii) Procedure. Twelve *Salmonella* biosensors were prepared as previously described using the commercial antiserum, and immersed in 110 ml of the filtered chicken exudate using the same technique as described under the longevity experiments. The plastic container, lid, Teflon biosensor retention block, and forceps for immersing the biosensors in the exudate were autoclaved prior to use. The container was sealed and refrigerated at 4°C and temperature monitoring was commenced as previously described. At select intervals of 24, 48, and 72 hours, 4 sensors each were removed aseptically in a microbiological safety cabinet and tested using *S. typhimurium* test solutions prepared as previously described.

Scanning electron microscopy analysis of biosensor. Physical characterization of the biosensor at the microscopic level was accomplished using scanning electron microscopy (SEM) analysis (Zeiss, DSM 940). The protocol for SEM analysis of monolayers and bacteria are based on those described by Hoppert and Holzenburg (43). The biosensors were first air-dried at room temperature in a sealed petri dish by elevating them over a small layer of desiccant using wooden applicator sticks. After 24 hours, the sensors were

mounted onto aluminum stubs with iron-based adhesive to insure proper conductance. The sensor-stub assembly was sputter-coated under 0.02 mbar Ar gas pressure (Pelco Sputter Coater, SC-7) with a 60:40 gold/palladium mixture at 30 mA for 1 minute. The sputter coating was then repeated to insure adequate coating of the sensor's top and bottom. The biosensors were examined and photographed at 10 kV. Micrographs of the specimens were captured digitally using a Digital Image Transfer Recognition Program (Zeiss), and processed with Adobe Photoshop 4.0.

Statistical analysis of data. The data derived from the experiments was analyzed using Microcal Origin 5.0 and Microsoft Excel statistical spreadsheet software. Acceptable performance of the biosensor was defined based on the accuracy and slope (sensitivity) of the linear portion of the dose response curve analyzed by least-squares regression. The ability of the biosensor to perform at a 0.05 level of confidence was determined. The voltage responses for each individual biosensor tested were plotted, and the mean value of a steady-state level for each voltage response was determined. The mean voltage responses were plotted against the logarithmic bacterial concentration of the test solutions in order to determine the slope of the voltage response (sensitivity) and the regression coefficient by regression analysis.

RESULTS AND DISCUSSION

(i) Determination of the functional longevity of the biosensor after immersion in raw chicken exudate. The working lifetime of the *Salmonella* biosensor was determined through the preparation, immersion (in chicken exudate), and testing of 136 sensors with five longevity experiments conducted under differing conditions of time and temperature.

a) Master the construction of the biosensor and operation of the microbalance to assay the sensor.

Thesis research involved the fabrication of 307 biosensors by immobilizing polyclonal *Salmonella* antibody monolayers to highly sensitive TSM quartz crystals to produce a piezoelectric biosensor capable of rapid, accurate, and sensitive detection of bacteria. Additionally, 56 checkslides were prepared using HNO_3 etched glass slides through the same patent-pending technology, allowing parallel observation of biosensor-exudate and antigen-antibody interaction.

In order to prepare a proper antibody monolayer for biosensor deposition, surface area/surface pressure isotherms were prepared as described by Gaines (32) and elasticity was calculated for the commercial and AU produced antibodies. For isotherm measurement, the monolayer was compressed into the known surface area of the trough at the subphase surface. As compression proceeded, the surface pressure increased causing the molecules of antibody and phospholipid to move closer together in a more

orderly fashion. The hydrophobic tails and hydrophilic heads of the phospholipids align, while the molecule of antibody orients itself within the monolayer so that its antigen binding sites were exposed. The changes in the surface pressure, as measured by the surface balance Wilhelmy plate, were derived from the differences between the surface tension of the water and the surface tension of the film.

The surface pressure measurements for each antibody used in biosensor preparation were performed and plotted against the mean molecular area of the film (Fig. 15). The isotherms for monolayers with antibodies were plotted together with the isotherm for an arachidic acid containing no antibodies. Surface pressure-area isotherms reflect both the structures of the molecules in the monolayer and the interaction between them. Compression of the monolayer eventually collapsed the monolayers, resulting in the sharp breaks seen for each isotherm. This collapsing point corresponds to the highest pressure at which the monolayer can be compressed without a detectable movement of the molecules out of the interface (33).

The elastic property of the antibody-phospholipid monolayer specifically describes the immobilization of the antibody in the film and its two dimensional compressibility, and is a function of the monolayers surface pressure as determined by its respective isotherm. The elasticity is calculated through the equation:

$$E = -\mathrm{A}\left(\frac{\delta\pi}{\delta \mathrm{A}}\right),$$

where A = area of the monolayer = $(450 - 1) \times 150$ [mm^2], π = surface pressure. The elastic property of the film provides a more lucid characterization of the antibody interaction with the phospholipid monolayer and describes the best pressure at which to

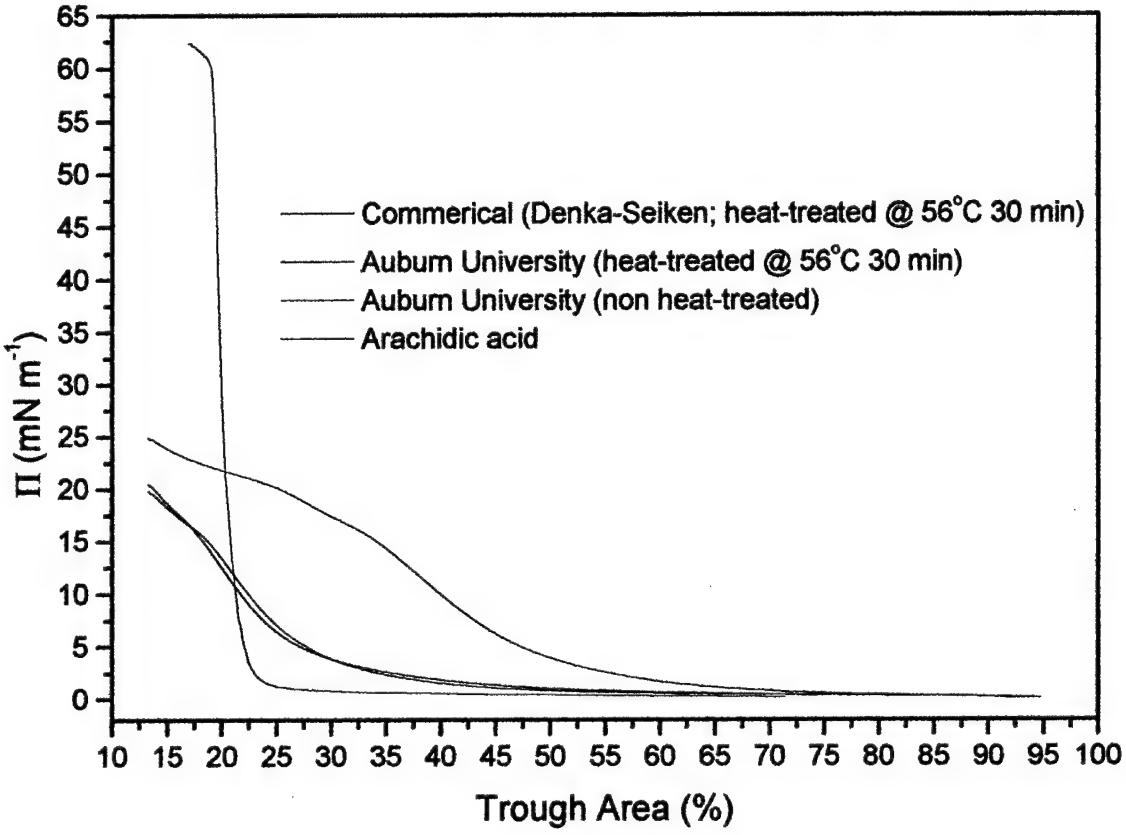

FIG. 15. Surface pressure isotherms of monolayers as a function of compression at 20°C.

compress the monolayers for optimal packing and transfer to the substrate. When plotted against the surface pressure measurements from the respective isotherm (Fig. 16), an optimal elasticity pressure is derived (Table 1). Again, the antibodies were plotted against a reference arachidic acid, which demonstrates characteristic elastic properties of a monolayer without antibodies. The maximum elasticity is higher than one found for pure protein monolayers (~10 mN/m at $A \approx 1$ m^2mg^{-1}) (25), and it agrees well with those found for cell membrane protein monolayers (9). When compared with pure protein films, the higher values of elasticity of lipid-protein monolayers indicate a better stability (26). Using this data, it was decided that the constant surface pressure at which to compress the antibodies would be 24 mN m^{-1}.

Monolayer transfer to the crystal sensors and checkslides was accomplished by Y-type LB film deposition for a hydrophilic substrate as described by Gaines (34). The monolayer is immobilized on the surface of the substrate both as it is raised out of the subphase through the monolayer and as it is lowered into the subphase through the monolayer. The transfer ratio (TR) was recorded for each monolayer. The TR is a numerical indicator of the amount of monolayer transferred to the substrate and calculated by the data processor of the KSV 2200 system through the formula:

$$\frac{\textit{Area of monolayer transferred to surface of the substrate}}{\textit{Total area of the substrate}}$$

where, the area of monolayer transferred to the surface of the substrate would be the decrease in the total available area of the monolayer film due to transfer during one

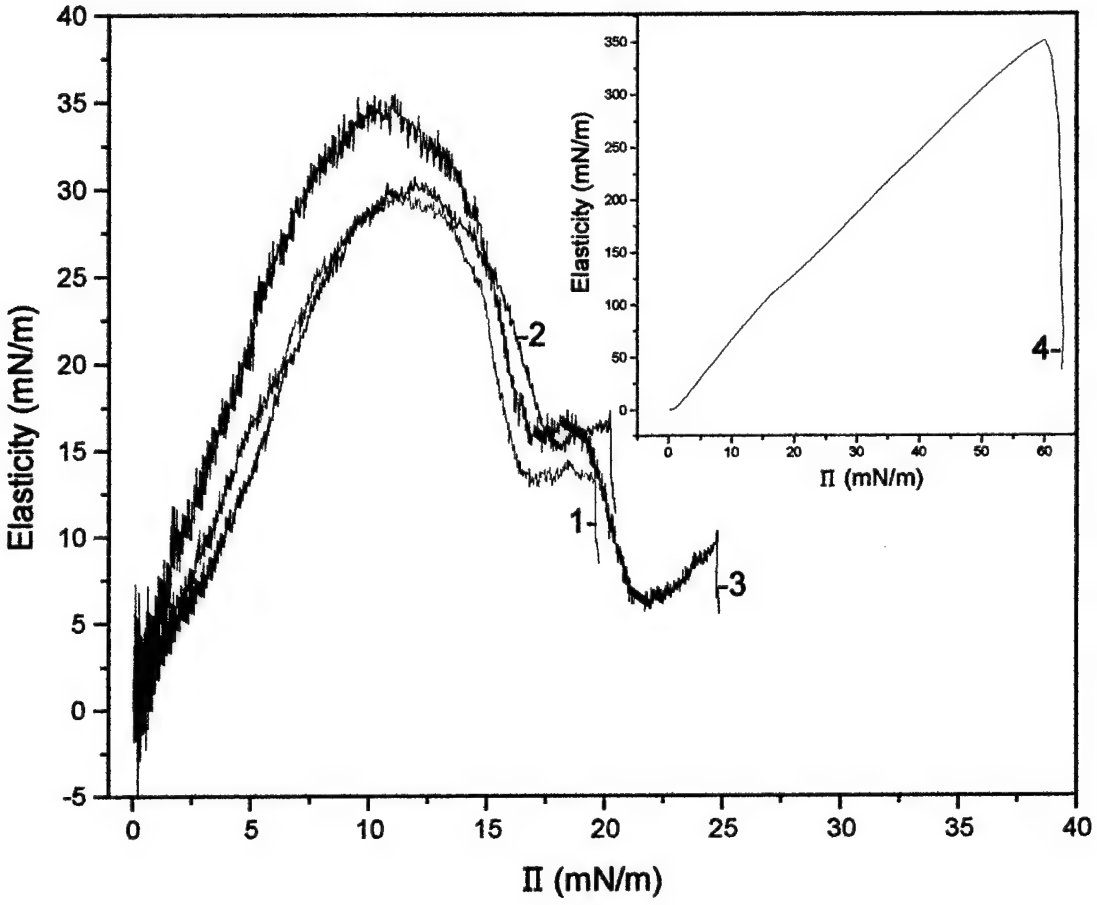

FIG. 16. Elasticity of monolayers as a function of surface pressure at 20°C. 1. Commercially prepared antiserum (Denka Seiken Co., LTD, Tokyo Japan; heated at 56°C for 30 minutes); 2. Auburn University prepared antiserum (heated at 56°C for 30 minutes); 3. Auburn University prepared antiserum (unheated). Inset: 4. Arachidic acid, pH corrected to pH 10.0 (courtesy Captain Mark Hartell, Auburn University).

TABLE 1. Optimal surface pressure and elasticity of monolayers at 20°C

Antibody Isotherm[e]	Surface Pressure (mN/m)	Elasticity (mN/m)
2[a]	11.96	30.70
1[b]	11.23	29.88
3[c]	11.11	35.40
4[d]	59.93	351.60

[a] Auburn University Poultry Science, heated at 56°C for 30 min.
[b] Denka Seiken Co., LTD., Tokyo, Japan; heated at 56°C for 30 min.
[c] Auburn University Poultry Science, unheated.
[d] Arachidic Acid at 20°C; pH corrected to 10.0.
[e] Antibody isotherm as shown in figure 16.

successive dipping of the substrate through the monolayer. The dipping area of the substrate is the effective diameter of 2 quartz crystal sensors (area of 4 crystal sensors placed back-to-back in the holder) multiplied by the depth of the immersion. The area does not include the crystal portion within the holder above the subphase. For a square object of identical length and height, the TR scale ranges from 0 to 1.0. A TR higher than 1.0 is an indicator that the layer was wrinkled during deposition. A TR lower than 1.0 evidences that the layer is only partially deposited. For the ideal deposition the TR = 1.0. Representative depositions of the three types of antiserum used in this work are compared to arachidic acid in figure 17. The arachidic acid (4) demonstrates a cumulative deposition TR of 6.7 out of 7.0 possible in 7 layers deposited, equating to near-perfect assembly of monolayers. Heat-treated antisera, both commercial (1) and non-commercial (2), show that 27 layers (up and down movements) were required in order to deposit the equivalent of 7 complete monolayers and indicates that TR for the process was about 0.4. Therefore, perfect overlapping monolayers were not expected to be found on the substrate, but rather overlapping patches of monolayer were deposited. Deposition of the non-commercial, non heat-treated antisera showed even smaller values of TR equal to 0.2. Therefore, this antiserum was not chosen for sensor preparations. Monolayers with a high TR were used for biosensors in this work.

b) Definition of the acceptable level of biosensor performance after immersion in order to have a minimal confidence level of correct functioning of the biosensor.

Pathirana et al. (66), previously discussed the criterion for acceptable level of biosensor performance. Figure 18 shows the sensor response curves obtained by exposing the sensor to buffer solutions containing different concentrations of the bacteria.

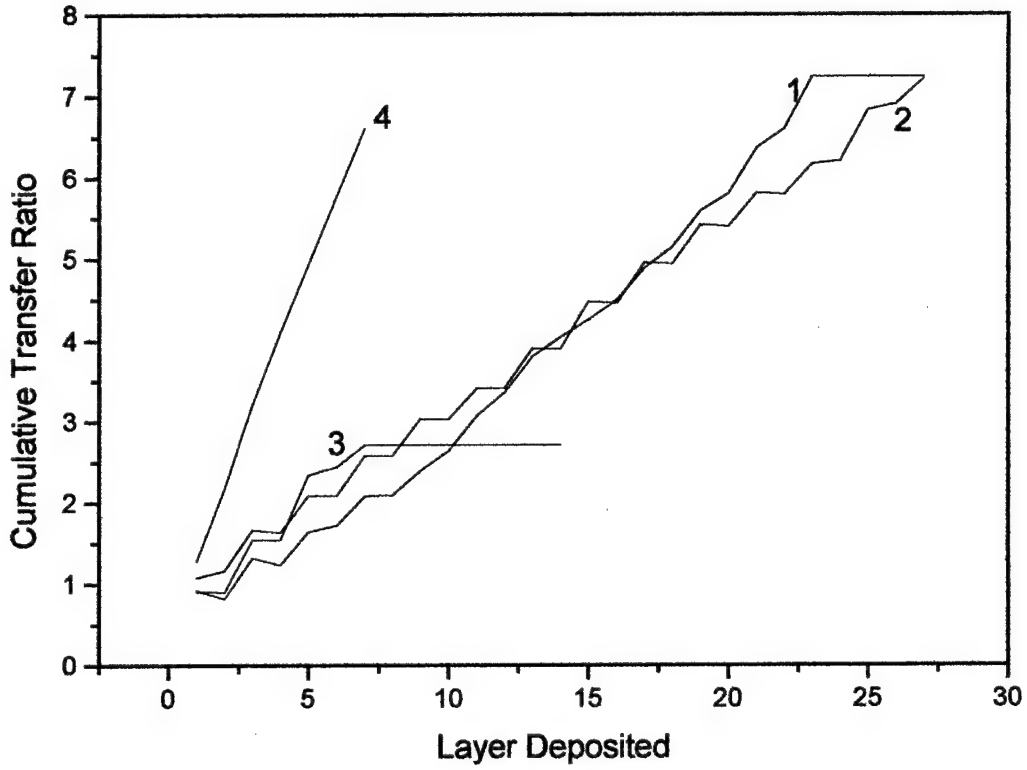

FIG. 17. Accumulative deposition of monolayers onto a sensor crystal substrate as a function of the Transfer Ratio at 20°C. 1. Commercially prepared antiserum (Denka Seiken Co., LTD, Tokyo Japan; heated at 56°C for 30 minutes); 2. Auburn University prepared antiserum (heated at 56°C for 30 minutes); 3. Auburn University prepared antiserum (unheated); 4. Arachadic acid, pH corrected to 10.0.

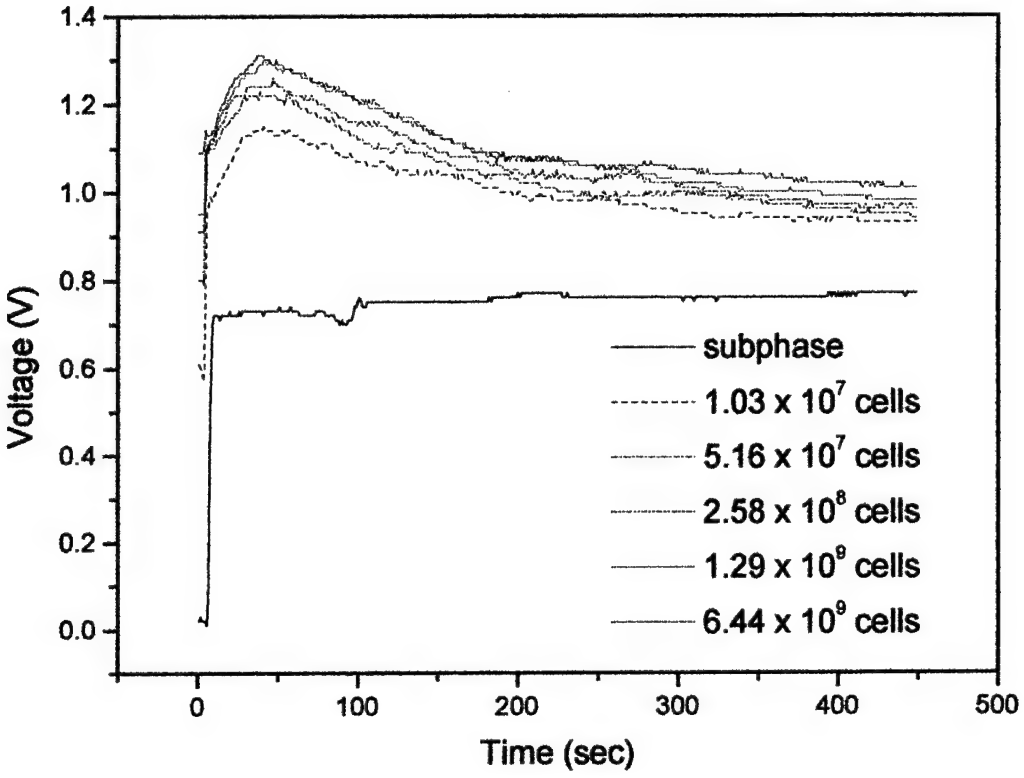

FIG. 18. Steady state voltage responses characteristic of a quintessential *Salmonella* biosensor tested with *S. typhimurium* test solutions.

From their research, they determined that the initial time of the response (τ_1=8±1 sec) does not depend on the bacterial concentration. As well, they determined that for each bacterial concentration, the sensor signal approaches a steady-state value, corresponding to that concentration within 100 sec (τ_2= 79±20 sec). The response curves are distinguished from previous reports (8, 9, 16, 17, 21) for pathogen detection by the fast reaction, the attainment of a steady state, and very low non-specific binding. Pathirana et al. (66), attribute these properties to the accuracy of the LB technique, the purification of antibodies during the process of immobilization, and the placement of the antibody in a compatible environment. It has been reported that 17-90 minutes were needed to measure bacteria binding by different acoustic wave biosensors (65, 70, 104).

In figure 19, the mean values of the steady-state output sensor voltages are plotted as a function of bacteria concentration from 1.03×10^7 to 6.44×10^9 cells ml^{-1}. The dose response is linear over 3 decades of bacterial concentration ($r \geq 0.98$, $p < 0.001$). The sensor sensitivity, measured as a slope of the linear portion of the dose response, is 25.8 mV $decade^{-1}$ of *Salmonella* concentration, based on experiments from 285 sensors. Pathirana et al. (66), criteria for acceptable performance of a biosensor were defined based on the accuracy and slope (sensitivity) of the linear portion of the dose response curve analyzed by least-squares regression. The biosensor performance was considered as acceptable if parameters of linear fitting met the following two criteria:

1) Regression coefficient, $r \geq 0.98$;

2) Sensitivity (linear portion of the dose response slope as a function of the biosensor voltage response) $\geq$ 10 mV $decade^{-1}$ of bacterial concentration.

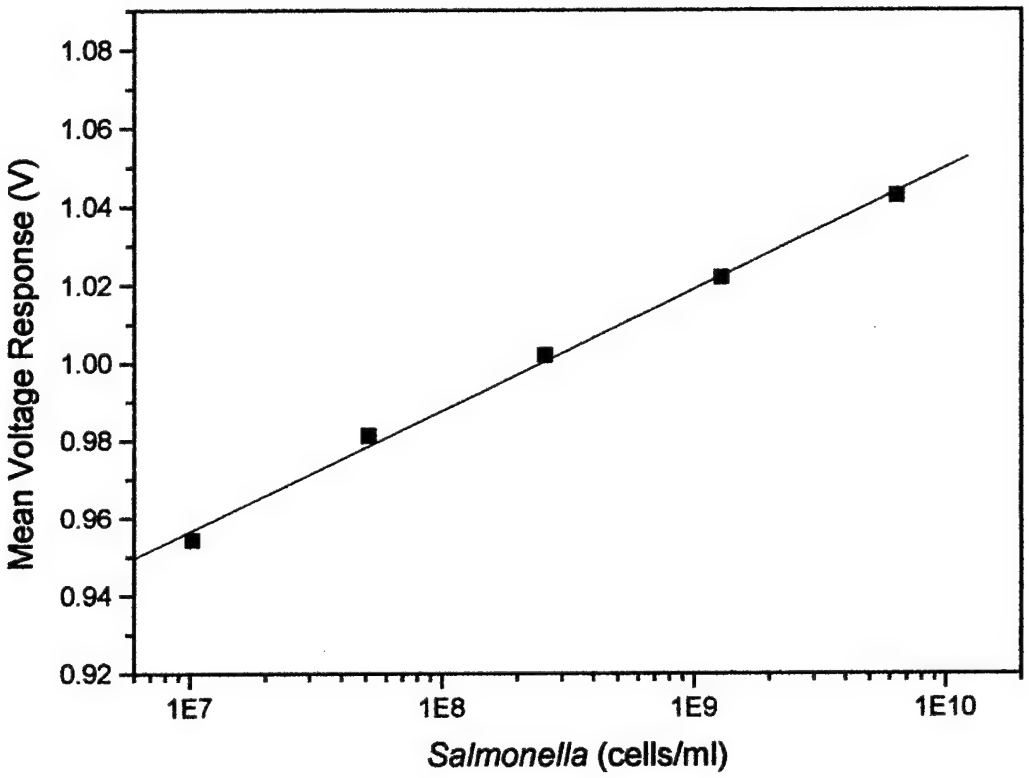

FIG. 19. Mean values of steady-state biosensor voltage responses as a function of logarithmic *S. typhimurium* test solutions. Overall, the dose response was confirmed through 4 decades of bacterial concentration.

Pathirana et al. (66), experimentation was based on 112 biosensors in which the dose response was linear ($r \geq 0.98$) over 5 decades of *Salmonella* bacteria with a sensitivity of 18.5 ± 5 mV decade^{-1}. To evaluate these criteria, 52 control biosensors were prepared and tested without the aging in solutions. Regression analysis shows that 32 of 52 met the minimum acceptable criteria with a mean sensitivity of 61.2 ± 9.2 mV decade^{-1}. This yields 62% of the prepared control biosensors passing the above criteria. The mean sensitivity of the control biosensors was found to be more than three times higher than those prepared by Pathirana et al. (66), whose sensors passed the minimal acceptable criteria at a rate of 40%.

c) Determination of the performance of the biosensor after immersion in chicken exudate for various durations and temperatures.

Longevity experiments were conducted within a refrigerator or incubator under controlled temperatures and monitored by thermistors. The thermistors were calibrated as described in the Methods and Materials section and results were fitted with sigmoidal calibration curves (Figs. 20, 21). During the longevity experiments, the container with the immersed biosensors was placed in the respective appliance and thermistor resistance was recorded every minute with the Scopeview program. The values of resistance were then converted to temperature (°C) by using the temperature calibration curves for the respective appliance. Recordings of immersion temperatures during longevity experiments are shown in figure 22. Comparison of the immersion temperatures during longevity experiments (Table 2) indicates that the sensors environmental temperature was most stable at room temperature immersion (23.22°C, SD = 0.60, $\delta = 0.03$) due to

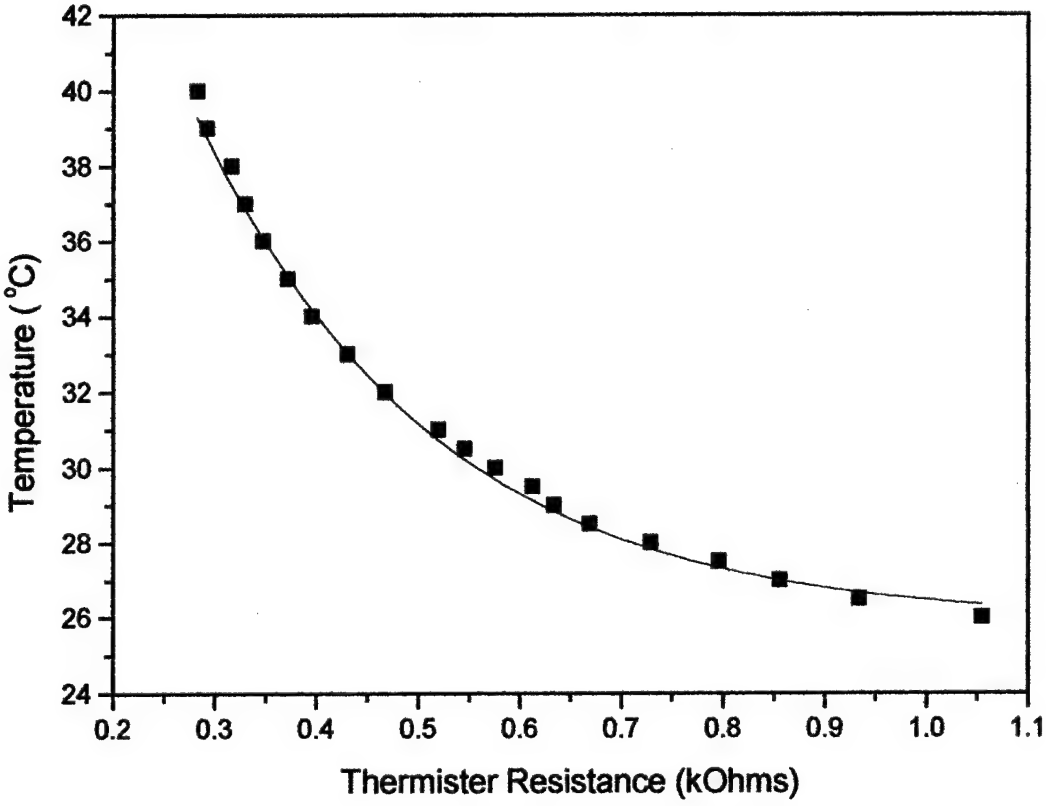

FIG. 20. Calibration curve of a thermistor showing temperature as a function of ohmic resistance under incubated conditions. Boltzman curve fitted; chi square = 0.0943.

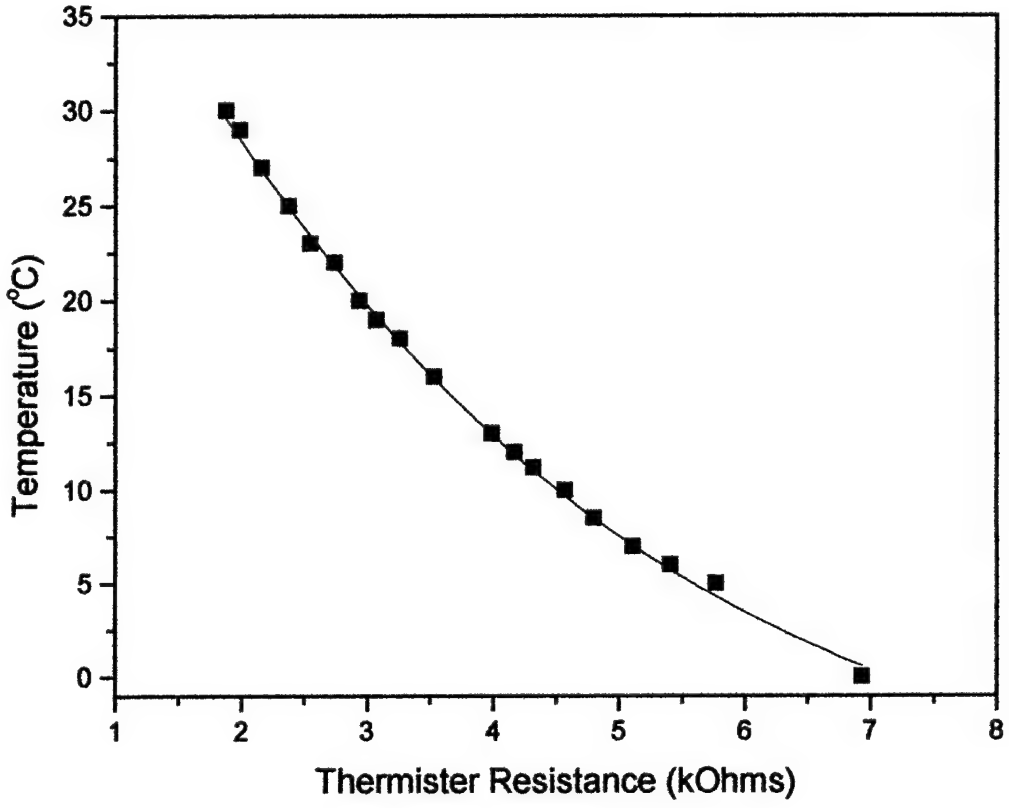

FIG. 21. Calibration curve of a thermistor showing temperature as a function of ohmic resistance under refrigerated conditions. Boltzman curve fitted; chi square = 121.06.

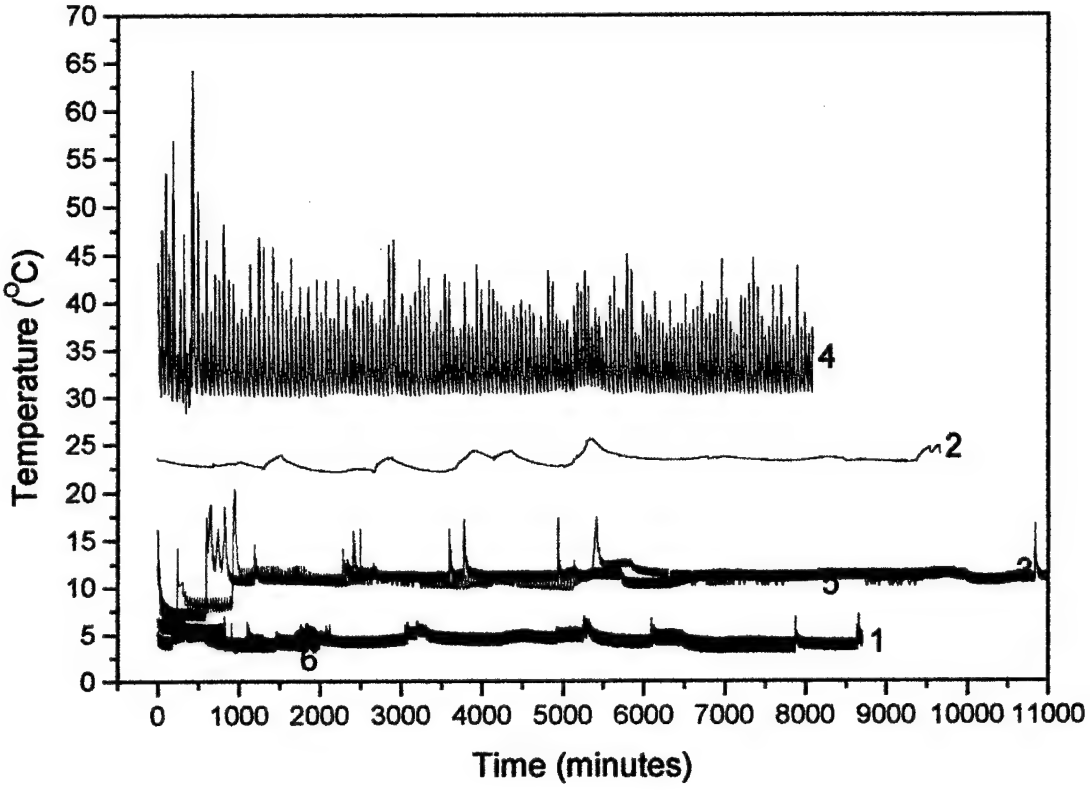

FIG. 22. Temperature monitoring of immersed biosensors during longevity experiments. 1. n = 8706, mean = 4.38°C; 2. n = 9661, mean = 23.23°C; 3. n = 15241, mean = 11.02°C; 4. n = 8094, mean = 33.72°C; 5. n =9732, mean = 10.72°C; 6. n = 2127, mean = 4.73°C (experiment 2). The incubator's (4) large deviation is a result of the instability of the temperature control apparatus.

TABLE 2. Temperature stability of biosensor preservation system

Preservation System	Mean Temperature	sd (yEr±)	n	δ	δ (%)
R[a]	4.38	0.63527	8706	0.14	14.50
R[a]	10.72	1.2077	9732	0.10	11.27
R[a]	11.02	1.05431	15241	0.10	9.56
R[a]	23.22	0.60398	9661	0.03	2.60
I[b]	33.72	3.49464	8094	0.10	10.36

[a] Refrigerator.
[b] Incubator.

decreased compressor activity, as compared to one needed to maintain cooler temperatures of 4°C or 11°C. Regression analysis determined that the stability of the refrigerated environment was dependent upon the temperature at which the system was maintained (Fig. 23).

Testing of biosensors was performed using a quartz crystal microbalance apparatus by measuring the change in the resonance characteristics of the biosensor. An unloaded crystal, excited into mechanical perturbation, vibrates in the thickness shear mode (TSM) at a frequency of approximately 5 MHz (56). The addition of material and changes in near surface viscoelasticity of the crystal surface will change the frequency at which the quartz crystal oscillates (48, 49, 58, 60, 65, 68, 69, 84). The voltage output from the Maxtek acoustic wave device is directly related to the resonance frequency of the quartz crystal sensor. Changes in the resonance frequency of the quartz crystal sensor were used to monitor the binding of bacteria to the sensor surface. The observed changes in the resonance frequency of the quartz crystal sensor during binding of bacteria is hypothesized to be due both to viscoelastic changes of the LB film-bacteria-near surface fluid media. The mass change is probably associated with the binding of bacteria.

The total TSM resonator admittance $\{Y(\omega)\}$ can be expressed by the equation:

$$Y(\omega) = j\omega C_o + 1/Z_m, \quad (1)$$

where ω is the angular excitation frequency,

$$\omega = 2\pi f, \quad (2)$$

C_o is the static capacitance of the quartz sensor, and Z_m is the motional impedance for the unloaded resonator.

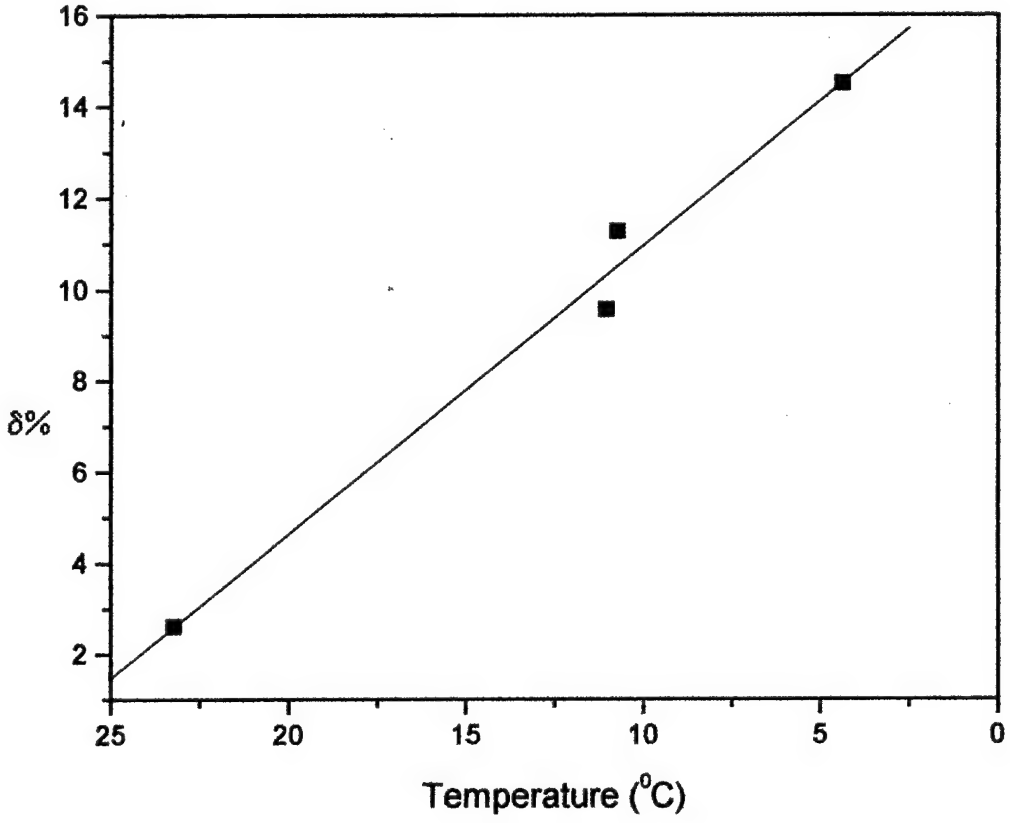

FIG. 23. Regression analysis showing that the stability of the refrigerator is a function of the temperature at which the system in maintained (r = 0.99237).

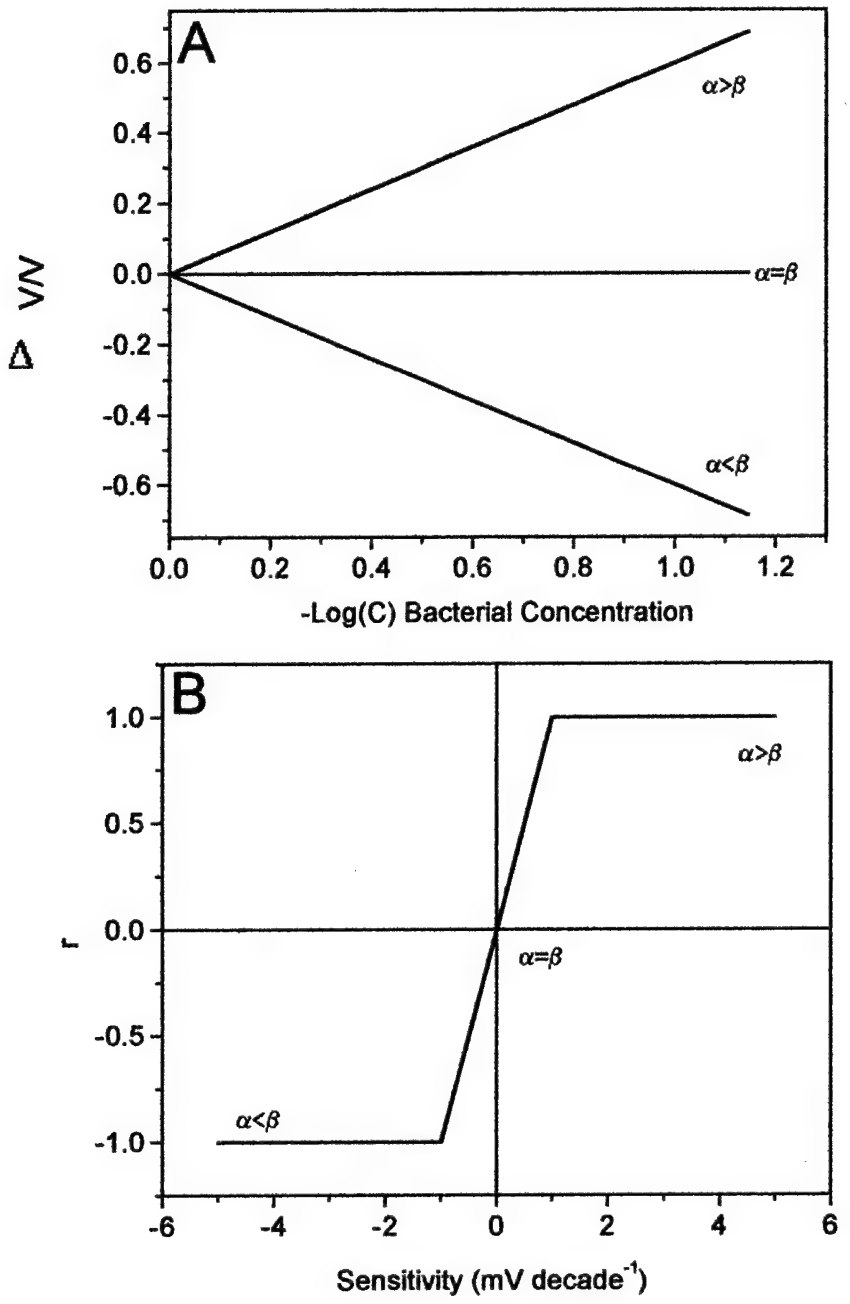

FIG. 24. Graph A: Relative change of the voltage (ΔV/V) is proportional to the logarithm of *Salmonella* concentration. Graph B: the regression coefficient is a function of the slope (sensor sensitivity) and can vary from –1 to +1, for different sensors with an inverse and direct concentration dependence, respectively.

$$Z_m = R + j\omega L + 1/j\omega C, \quad (3)$$

where R, L, and C are the resistance the inductance and capacitance of the motional arm in parallel with the capacitance C_o. The series resonant frequency f can be determined as

$$f = 1/2\pi\,(LC)^{1/2}. \quad (4)$$

When the resonator is loaded with solutions the resonant frequency changes due to small changes of parameters L and C. The differential of resonant frequency, df, can be calculated as following:

$$df = (\partial f/\partial L)dL + (\partial f/\partial C)dC, \quad (5)$$

where partial derivatives $\partial f/\partial L$ and $\partial f/\partial C$ can be calculated from equation (4):

$$\partial f/\partial L = -\,(f/2)/L, \quad (6)$$

$$\partial f/\partial C = -\,(f/2)/C. \quad (7)$$

Substituting partial derivatives with equations (6) and (7), and replacing the differentials df, dL, and dC with small changes of the values, we have:

$$\Delta f/f = -\,1/2 \times (\Delta L/L + \Delta C/C). \quad (8)$$

If binding bacteria increases the effective mass of the resonator, then the serial inductance (L), representing a mass in the equivalent-circuit model, should increase with the increase of bacteria concentration (5). On the other hand, binding bacteria increase the effective thickness of the resonator and, consequently, decrease the equivalent capacitance (C) of the circuit. Thus, we can speculate, that relative changes of the inductance and capacitance are linear functions of the bacteria concentration (C):

$$\Delta L/L = \alpha\log(C), \quad (9)$$

$$\Delta C/C = -\beta\log(C), \quad (10)$$

where coefficients α and β are independent of bacteria concentration.

From equations (8)-(10) we have:

$$\Delta f/f = - 1/2 \times \alpha(1-\beta/\alpha)\log(C). \quad (11)$$

The relative (V) shifts of the Maxtek PM 740 acoustic wave device are linearly proportional to the relative frequency shifts with a negative proportionality constant. Therefore we can replace $\Delta f/f$ with $-\Delta V/V$ in equation (11):

$$\Delta V/V= 1/2 \times \alpha (1-\beta/\alpha)\log(C). \quad (12)$$

Equation (12) indicates, that if the relative increase of the effective thickness is small compared to increase of the effective mass ($\alpha > \beta$), then the relative change of the voltage ($\Delta V/V$) is directly proportional to the logarithm of bacterial concentration (Fig. 24b, line $\alpha>\beta$). If the opposite is true, and $\alpha < \beta$, then $\Delta V/V$ is inversely related to the concentration of bacteria (Fig. 24b, line $\alpha<\beta$). If $\alpha = \beta$ then $\Delta V/V$ does not depend on the bacterial concentration (Fig. 24b, line $\alpha = \beta$):

$$\Delta V/V \sim 1/2 \times \alpha\log(C), \text{ if } \alpha>\beta, \quad (13)$$

$$\Delta V/V \approx 0, \alpha\approx\beta, \quad (14)$$

$$\Delta V/V \sim -1/2 \times \beta\log(C), \text{ if } \alpha<\beta. \quad (15)$$

When experimental dose response data are fitted by a linear regression analysis to a line $\Delta V/V = A + B\log(C)$, the slope of the line (sensitivity), B, and the regression coefficient, r, are calculated for each sensor. As shown in figure 24b, when $\alpha > \beta$, a good positive correlation at large slope (sensitivity) is also expected to be confirmed by a high regression coefficient ($r \approx 1$). If $\alpha < \beta$, the regression coefficient at a large negative slope tends to approach a large negative value ($r \approx 1$). At conditions, when $\alpha \approx \beta$, $r \approx 0$. Thus

the regression coefficient can be a function of the slope and can vary from –1 to +1, for different sensors with an inverse and direct concentration dependence, respectively.

Thus, bacterial binding can be monitored as a voltage output that increases proportionally with the thickness of bacteria deposited (Fig. 18). A steady state voltage response is reached as a proportional amount of bacteria first bind, then loosen themselves through the oscillation of the unit. When these steady-state voltage responses are plotted as a function of an increasing logarithmic bacterial concentration, and a linear slope is fitted (Fig. 19), the biosensor becomes an extremely accurate indicator of the bacterial concentration of a liquid applied to its surface.

During four longevity experiments, 112 biosensors and 28 checkslides were prepared and immersed in chicken exudate in four groups of 28 and 7, respectively, at temperatures of ~ 4°C, 11°C, 23°C, and 33°C. For each experiment; at intervals of 24 hours for 7 days, 4 sensors were removed for testing in quadruplicate, and 1 checkslide was removed for examination (Figs. 25, 26). The only exception to this routine was the 33°C experiment, which was stopped after 3 days due to severe denaturation of the chicken exudate (Fig. 27); all the sensors and slides remaining after 3 days were removed and tested or examined together.

Regression analysis of the initial experiments at 4°C, 11°C, 23°C and 33°C determined that only 5 of 104 biosensors met the minimum acceptable criteria of positive correlation and sensitivity. Three of these sensors were from four tested after immersion at 4°C for duration of one day. This gives a 75% success rate for the group of sensors immersed one day or less. No other biosensors in the four experiments fit the acceptable

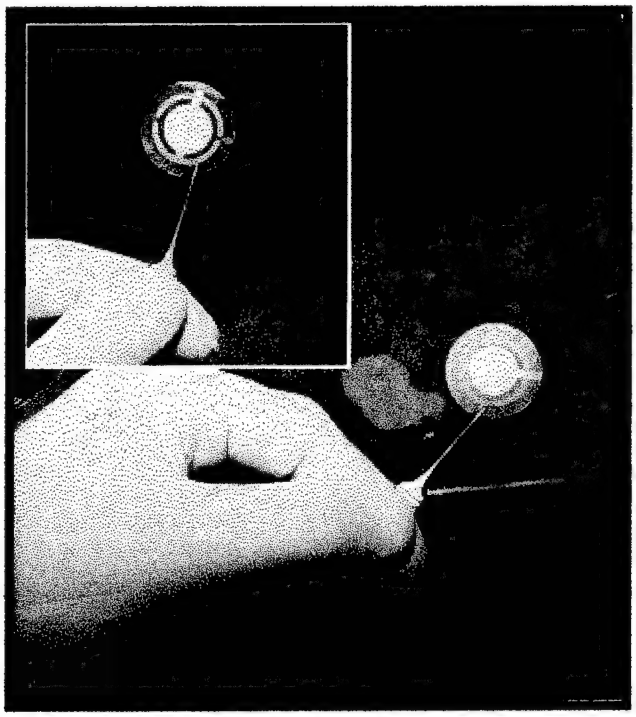

FIG. 25. Biosensor after removal from chicken exudate immersion for 7 days at 4°C. Denaturation of the exudate proteins has left a layer of material on top that was easily rinsed off with subphase. Inset: Biosensor after rinsing.

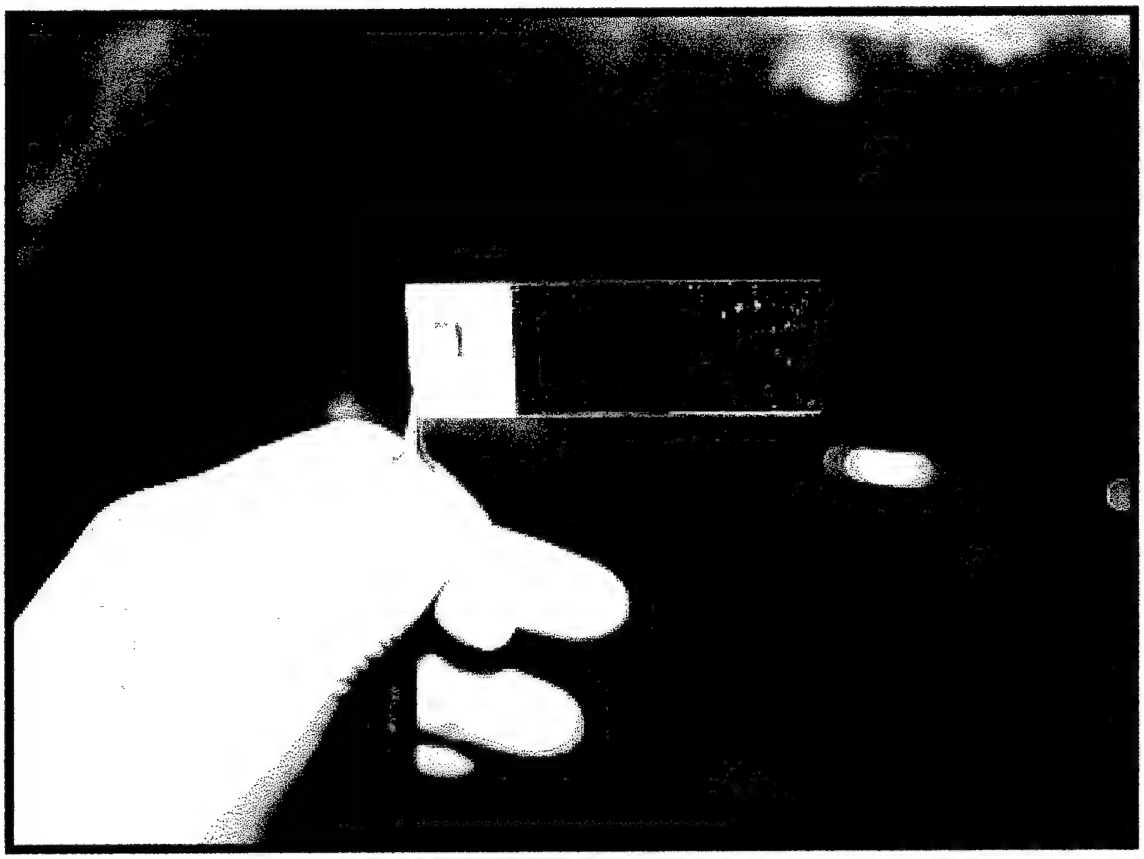

FIG. 26. Checkslide post-immersion in chicken exudate for 7 days at 4°C. The slide has been rinsed with subphase and notably exhibits a hazy area and visible line of demarcation where monolayers were deposited (arrow) at the end of glass slide.

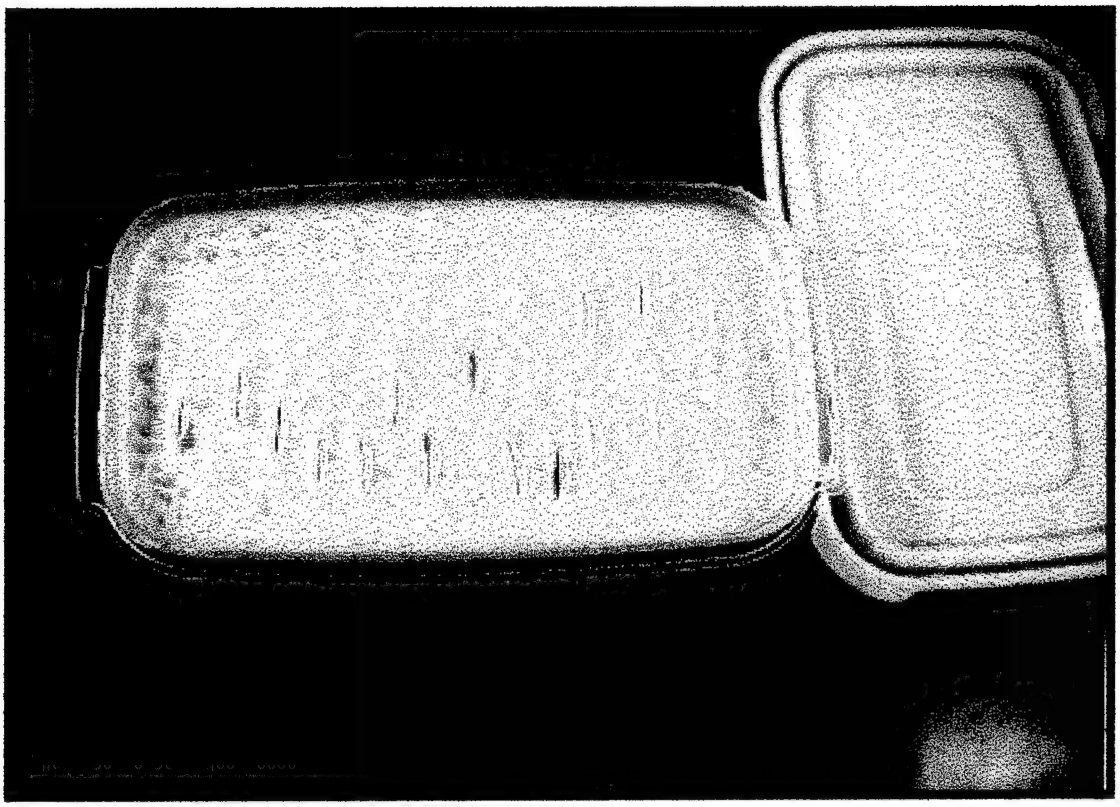

FIG. 27. Sensors in chicken exudate after immersion for 3 days at 33°C. The denaturation of the chicken exudate is evident and left the sensors in a globular mass. The sensors are in an upright, vertical position due to retention in the slotted Teflon block.

TABLE 3. Functional performance of biosensors post-immersion in non-filtered chicken exudate

Immersion (days)	Mean sensitivity response ± sd (yEr±) of biosensors post-immersion in chicken exudate at temperature (°C)[a]			
	4	11	23	33[b]
1	63.8 ± 37.2	4.7 ± 1.8	3.4 ± 5.2	13.9 ± 93.9
2	-17.6 ± 5.8	-8.7 ± 11.7	-3.3 ± 3.9	114.0 ± 322.7
3	3.1 ± 3.2	-0.3 ± 8.8	-8.2 ± 4.2	84.2 ± 284.4
4	1.1 ± 6.9	-1.9 ± 10.1	-1.2 ± 2.9	
5	1.4 ± 3.3	-4.6 ± 3.1	-9.1 ± 30.8	
6	8.6 ± 9.5	-9.9 ± 0.3	-6.0 ±18.1	
7	-0.7 ± 3.0	80.5 ± 139.9	-10.3 ± 4.6	

[a] Average of sensors tested in quadruplicate.
[b] After 3 days of immersion the remaining sensors were removed and tested due to denaturation of chicken exudate.

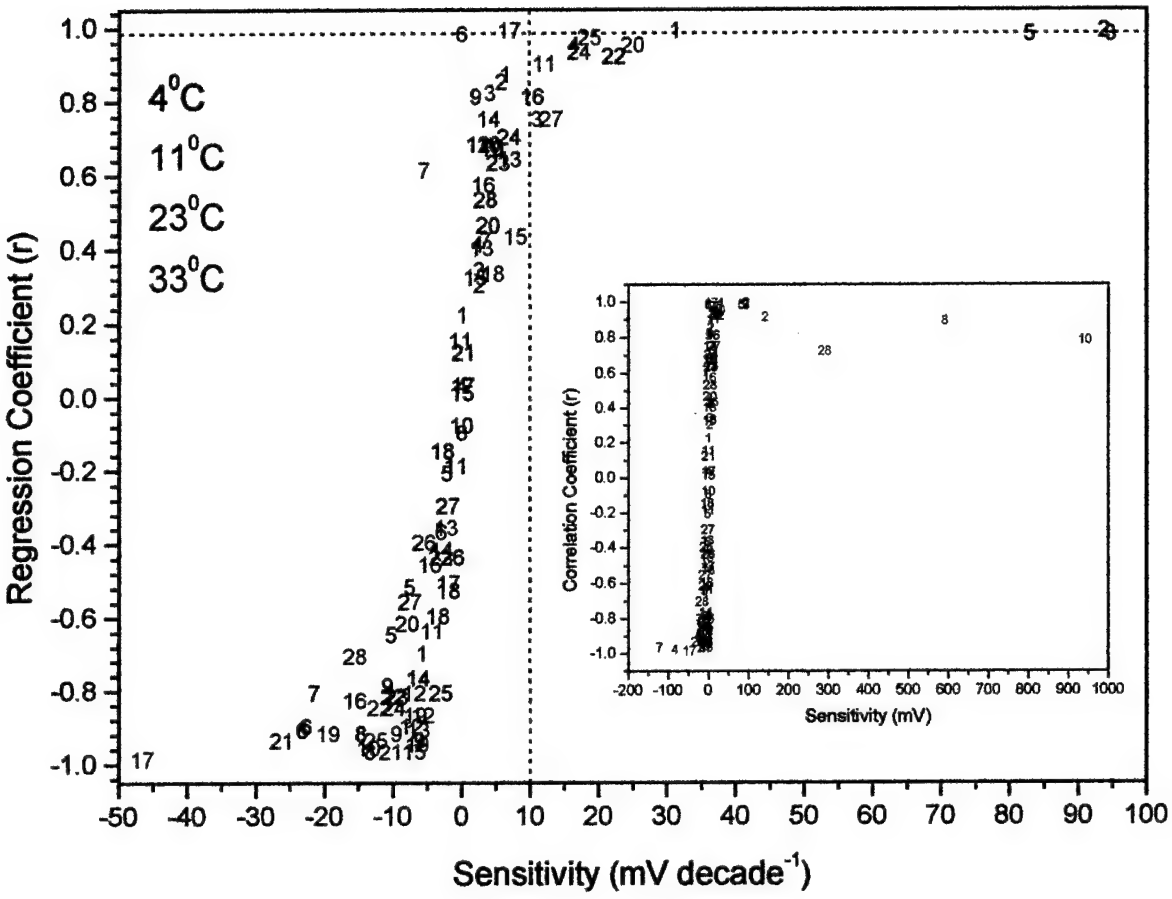

FIG. 28. The majority of biosensor test results for longevity experiments conducted between 4°C and 33°C fell between –50 and 100 mV decade^{-1} sensitivity. Only three of the sensors feel within the criteria. Axes at 10 mV decade^{-1} and 0.98 are the minimum acceptable criteria for a successfully performing biosensor. Inset: test results of all biosensors in immersion experiments. Biosensors for each experiment are shown by numerical designation.

criteria. Eight of the biosensors from the 33°C experiment were severely encrusted with denatured exudate and failed to allow testing. Table 3 and figure 28 summarize the data from regression analysis and shows that the sensors immersed at 4°C for one day had the only positive linear correlation > 0.98 and voltage response slope above 10 mV decade^{-1} (26.6-110 mV decade^{-1}).

The results from the 4°C experiment warranted a more timely evaluation to determine the functional longevity of the biosensor in hours instead of days, therefore, a fifth experiment was conducted by immersing 24 biosensors for a period of 48 hours duration at 4°C. 4 sensors were removed for testing in quadruplicate at intervals of 2, 4, 8,16, 32, and 48 hours. Regression analysis of experimental data confirmed that the functional duration of the biosensors was 32 hours post-immersion in chicken exudate at 4°C only (Table 4). 50-75% of the sensors tested for each temperature up to 32 hours met the minimum acceptable criteria for a successful performance ($r \geq 0.98$, sensitivity $\geq$ 10 mV decade^{-1}). None of the sensors for 48 hours duration performed at these limits. 12 of 23 overall (1 failed to reset to zero and was omitted statistically) performed at the acceptable level for a 52% sensor success rate overall.

Combining the data for the two 4°C immersion experiments gives us results for biosensors immersed for intervals of 2, 4, 8, 16, 19, 32, 45, 48, 66, 99, 120, 144, and 171 hours (Fig. 29). Between 32 and 45 hours post-immersion, the sensors will no longer function at the minimum acceptable performance level when tested. As well, there seems to correlation between immersion time and sensitivity; as the time immersed increases, the sensitivity decreases (Fig. 30).

TABLE 4. Functional performance of biosensors post 4°C immersion in non-filtered chicken exudate

Immersion (hours)	Regression coefficient (r) / sensitivity response (mV decade^{-1}) of biosensors[a]			
	1	2	3	4
2	**0.99 / 16.19**	**0.99 / 26.03**	0.52 / 9.55	0.59 / 4.98
4	**0.99 / 134.02**	**0.99 / 84.96**	**0.99 / 80.46**	[b]
8	0.95 / 7.53	**0.98 / 18.23**	**0.98 / 35.75**	0.96 / 21.20
16	**0.99 / 126.87**	0.87 / 4.63	**0.99 / 12.45**	0.97 / 5.62
32	**0.98 / 15.29**	**0.99 / 20.27**	**0.99 / 28.32**	0.76 / 6.64
48	0.92 / 8.57	-0.88 / -222.18	0.88 / 8.76	0.92 / 101.19

[a] Sensors tested in quadruplicate. **Bold indicates passing performance;** minimum criteria for acceptable level of performance is $r \geq 0.98$, sensitivity ≥ 10 mV decade^{-1}.
[b] Electrode was scratched; sensor could not be tested.

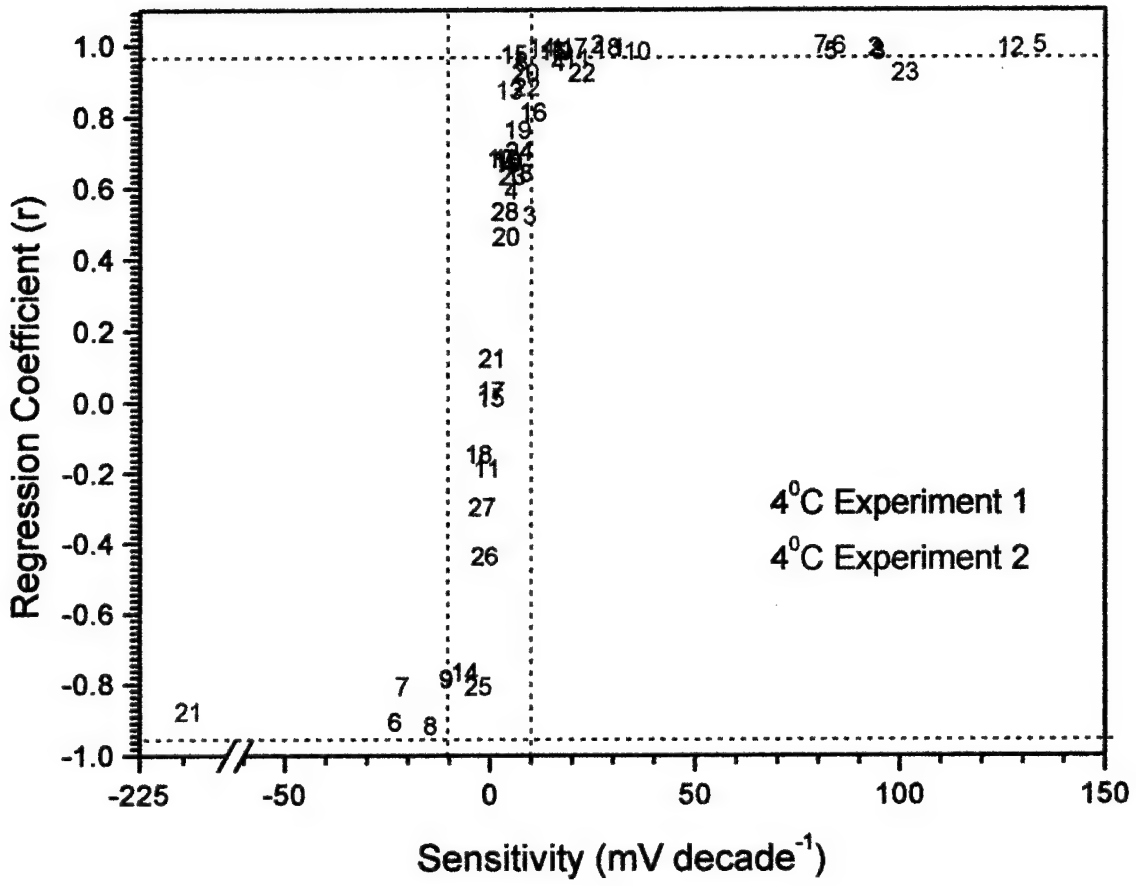

FIG. 29. Regression analysis results of two longevity experiments conducted at 4°C. Axes at 10 mV decade^{-1} and 0.98 are the minimum acceptable criteria for a successfully performing biosensor. Biosensors for each experiment are shown by numerical designation.

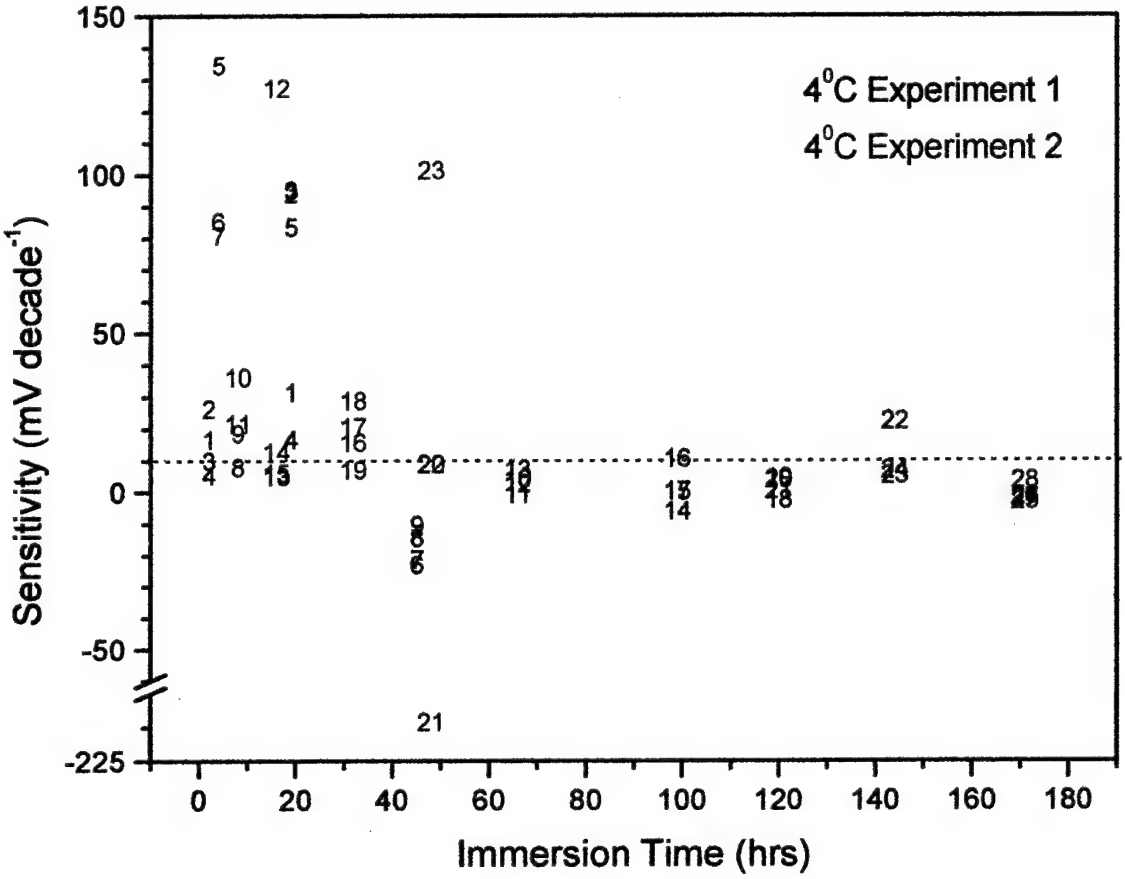

FIG. 30. Sensitivity results of biosensors from two longevity experiments conducted at 4°C as a function of the duration of immersion. Biosensors for each experiment are shown by numerical designation.

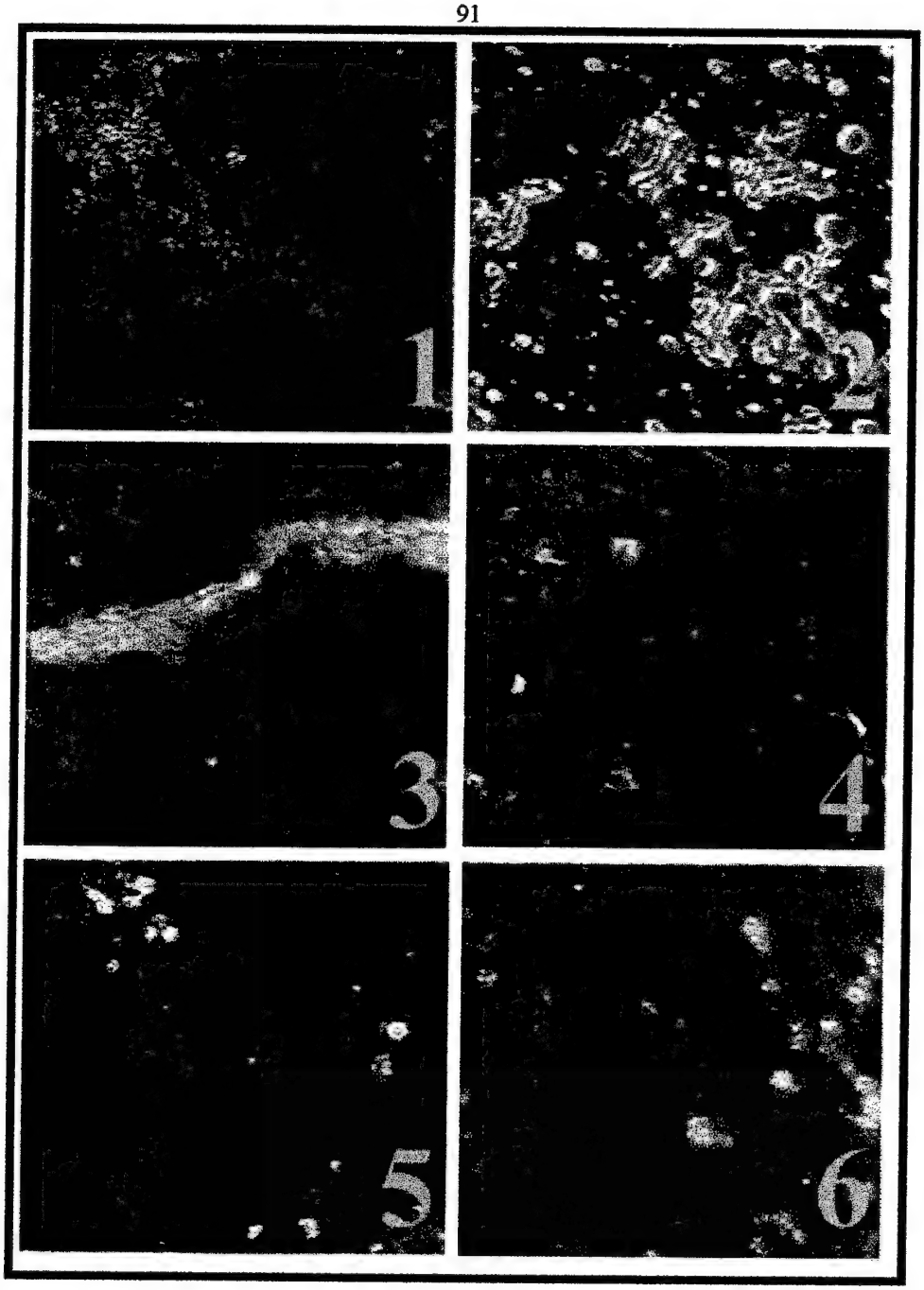

FIG. 31. Checkslides from 4°C longevity experiment show increased buildup of indigenous bacteria as the time of immersion in chicken exudate increases. In numerical order of 1-6: day 1, day 2, day 3, day 4, day 5, day 7. By day 7 (6), the slide was partially covered with bacterial remnants and extraneous material from the chicken exudate.

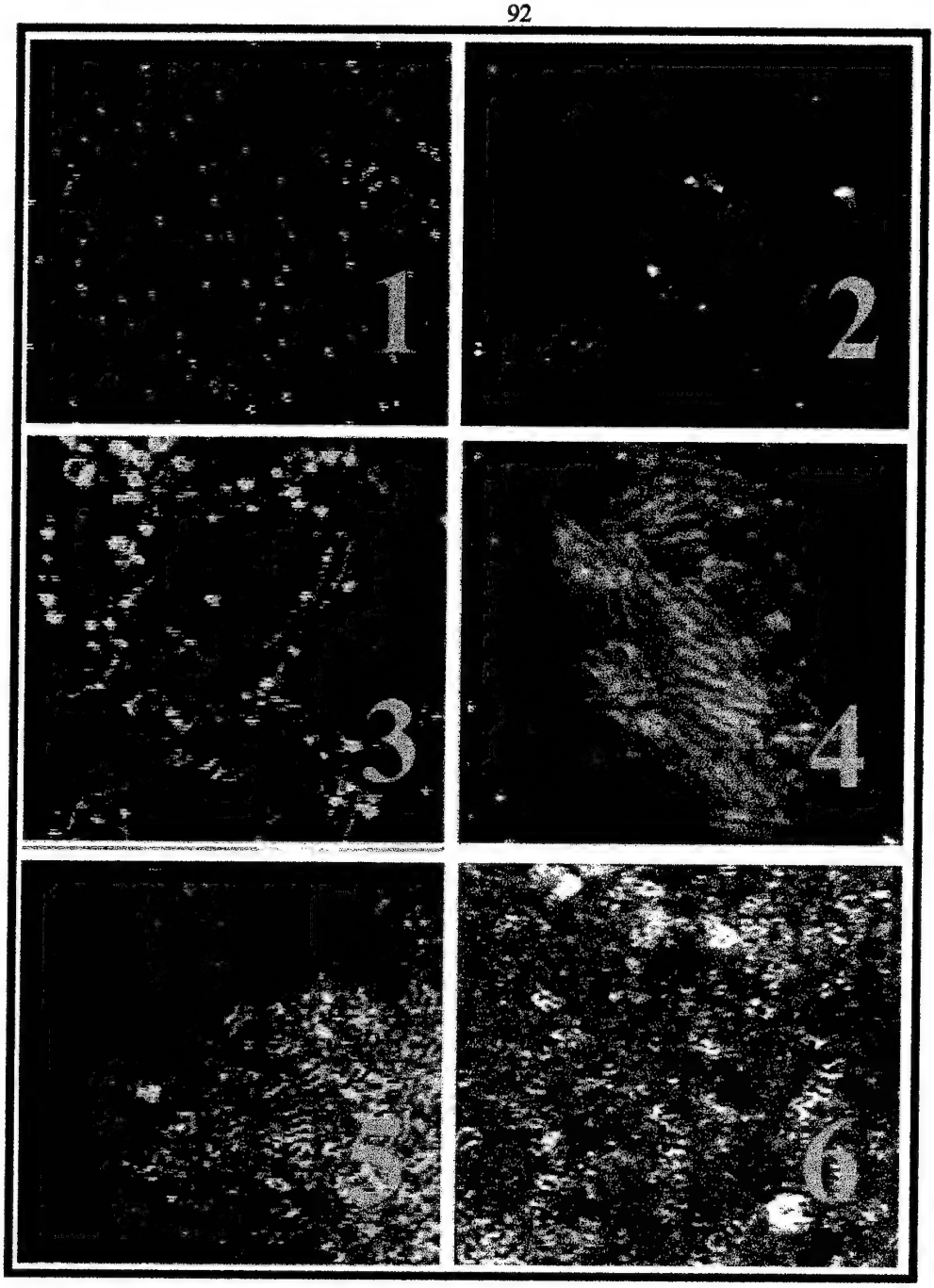

FIG 32. Checkslides from 11°C longevity experiment show increased buildup of indigenous bacteria as the time of immersion in chicken exudate increases. In numerical order of 1-6: day 1, day 2, day 3, day 5, day 6, day 7. By day 7 (6), the slide was totally covered with bacterial remnants and extraneous material from the chicken exudate.

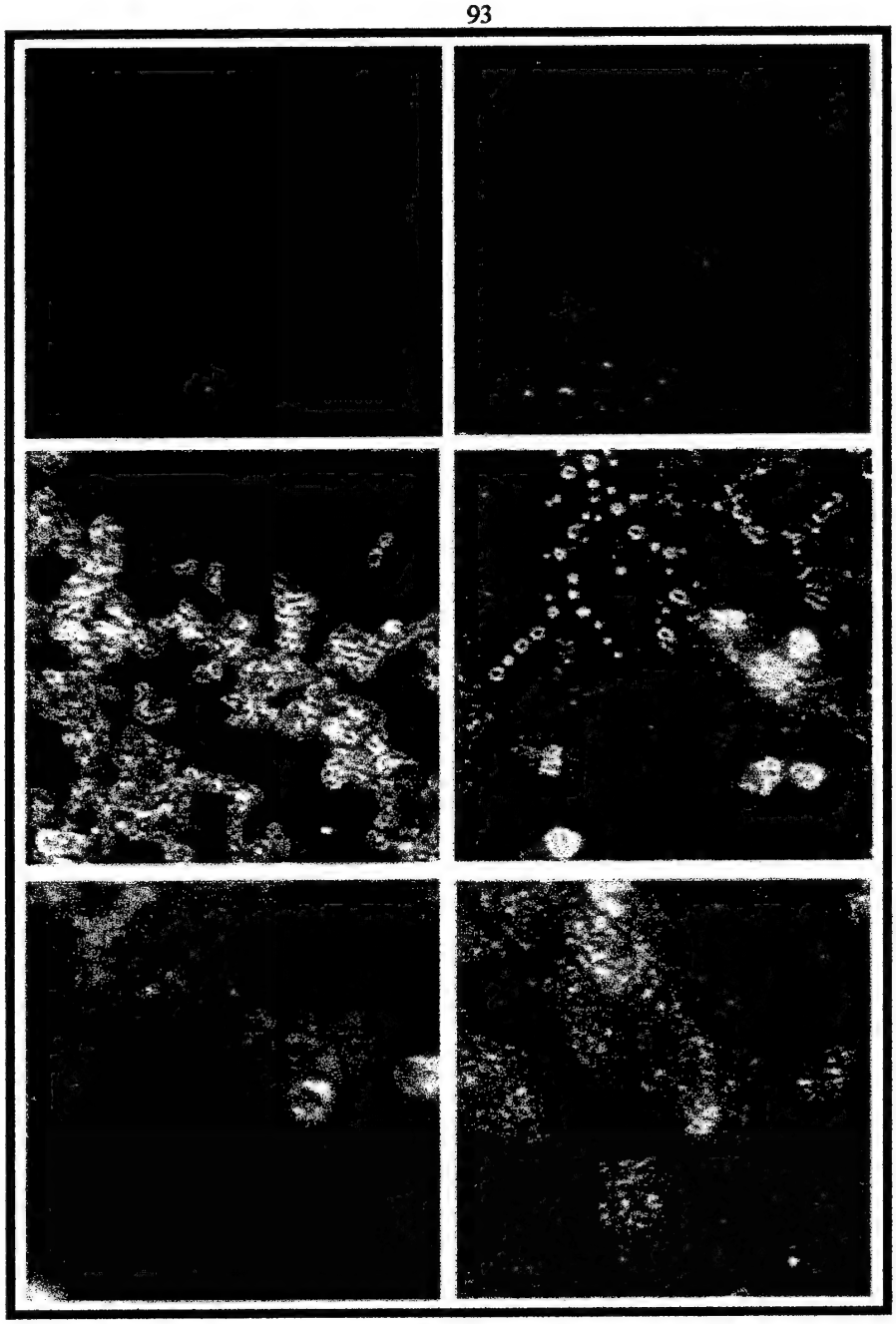

FIG. 33. Checkslides from 23°C longevity experiment show increased buildup of indigenous bacteria as the time of immersion in chicken exudate increases. In numerical order of 1-6: day 1, day 2, day 3, day 4, day 5, day 7. By day 7 (6), the slide was totally covered with bacterial remnants and extraneous material from the chicken exudate.

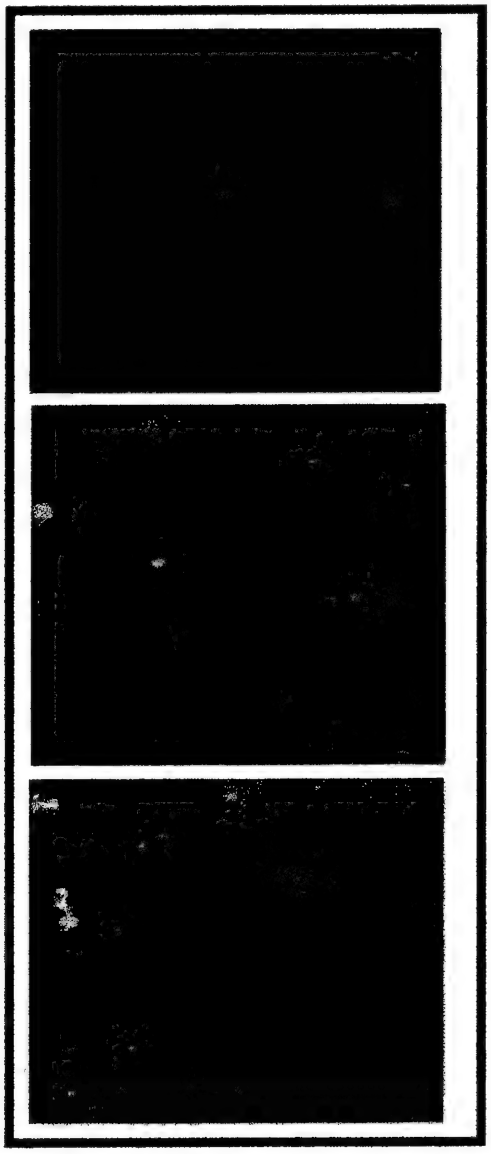

FIG. 34. Checkslides from 33°C longevity experiment show increased buildup of indigenous bacteria as the time of immersion in chicken exudate increases. In numerical order of 1-3: day 1, day 2, day 3. By day 2 (2), the slide was totally covered with bacterial remnants and extraneous material from the chicken exudate.

Microscopic examination of checkslides revealed extensive bacterial buildup on the monolayer after 2 days duration at 4°C, and an immediate buildup of bacteria on the monolayer at higher temperatures (Figs. 31-34). It is thought that this buildup of dead cells on the monolayer perhaps masks the binding sites and interferes or degrades the antibody binding ability of the sensor since buildup mirrors the decrease in functioning of the sensor. Testing with concentrated *S. typhimurium* revealed slight agglutination only on the checkslide removed after immersion for one day at 4°C; there was no agglutination noted on the other 27 checkslides during testing. QC checkslides tested concurrently with the immersed checkslides performed as expected, with some agglutination on each of the 28 prepared (Fig. 35).

(ii) Specificity testing of the biosensor. To confirm the ability of the biosensor to discriminate between different antigens, two different specificity experiments were conducted.

S. typhimurium versus E. coli O157:H7. Eight *Salmonella* biosensors were prepared and tested with either *S. typhimurium* or *E. coli* O157:H7 test solutions. Regression analysis (Table 5) ascertained that 2 of 4 biosensors tested with *S. typhimurium* met the minimum acceptable criteria of positive correlation and sensitivity. The other two sensors did not perform at the acceptable level, but demonstrated a steady state response signal to the *S. typhimurium* test solutions. None of the 4 biosensors tested with *E. coli* O157:H7 met the minimum acceptable criteria of positive correlation and sensitivity or showed a steady state response signal. These results confirm the specificity findings of identical *Salmonella* biosensors produced by Pathirana et al. (66), and parallel the high specificity of the *Salmonella* biosensors produced by Prusak-Sochaczewski and Luong

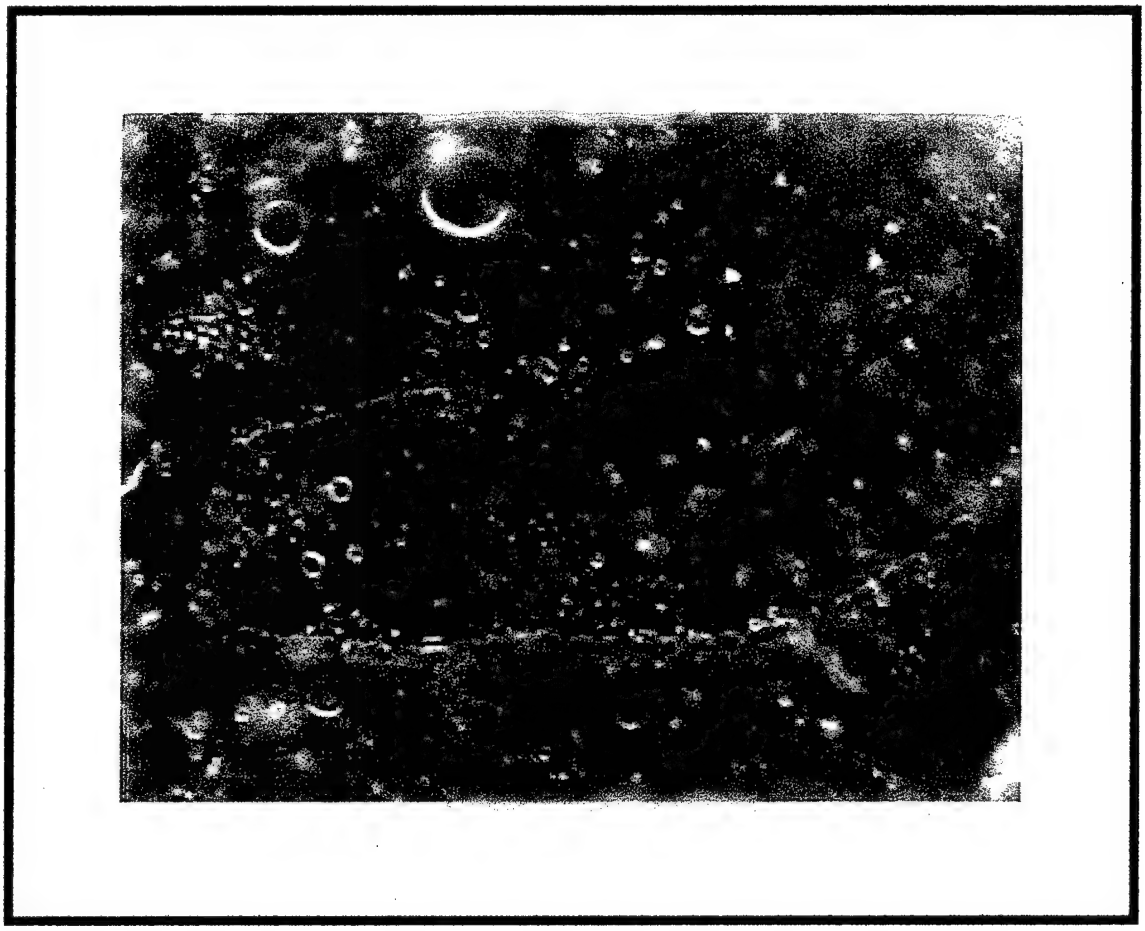

FIG. 35. Checkslide tested with *S. typhimurium* for quality control assurance. Photograph is comparable to SEM micrograph in figure 39 and shows *Salmonella* bacteria (arrow) binding via antibody interaction. Naesssens darkfield microscopy; magnification, ×1,000; bar = 5μm.

TABLE 5. Functional performance of biosensors in specificity testing

Test Solution[e]	Regression coefficient (r) / sensitivity response (mV decade^{-1}) of biosensors[a]			
	1	2	3	4
St[b]	**0.99 / 62.00**	**0.98 / 20.97**	0.96 / 35.58	0.96 / 143.39
Ec[c]	0.76 / 30.02	0.96 / 8.29	0.65 / 8.12	-0.60 / - 0.00
UnabsSt[b]	0.96 / 196.21	**0.98 / 20.41**	**0.99 / 25.49**	0.96 / 77.36
AbsSt[d]	**0.99 / 38.19**	0.83 / 44.87	**0.98 / 28.51**	0.84 / 18.32

[a] Sensors tested in quadruplicate. **Bold indicates passing performance;** minimum criteria for acceptable level of performance is r ≥0.98, sensitivity ≥10 mV decade^{-1}.
[b] Normally prepared *S. typhimurium* test solution.
[c] Normally prepared *E.coli* O157:H7 test solution.
[d] Blocked *S. typhimurium* test solution.
[e] Test solution designation based on database file name.

(68). As well, the exacting specificity of the sensor to *S. typhimurium* matches Ye et al. (103), findings that a piezoelectric crystal with immobilized anti-*Salmonella* spp. should react specifically with *Salmonella* cells only and bind them to the surface of the crystal.

Unabsorbed S. typhimurium versus absorbed S. typhimurium. Eight more *Salmonella* biosensors were prepared and tested with either blocked *S. typhimurium* test solutions or unblocked *S. typhimurium* test solutions using free *Salmonella* antiserum. Regression analysis (Table 5) ascertained that 2 of 4 biosensors tested with the normal, unblocked test solutions met the acceptable performance criteria. Again, this confirms the findings of Pathirana et al. (66) and the Ye et al. (103) as noted previously. Two of the 4 biosensors tested with the blocked *S. typhimurium* also met the acceptable criteria. This was probably due to a prozone effect, where the binding sites of the cells outnumbered the antibody in solution, allowing further binding of the *Salmonella* at the sensor surface. All eight sensors demonstrated steady state response signals but did not meet the performance criteria.

(iii) Increasing the working longevity of the biosensor. To investigate the possibility of increasing the working longevity of the biosensor, three experiments were conducted. The first experiment investigated the possible causes of monolayer degradation through filter sterilization of the chicken exudate. The second investigated sensor reusability by reversing bacterial binding. The third examined the effects that differing antibody preparation had on preparing a more efficient monolayer.

a) Filtration testing.

TABLE 6. Functional performance of biosensors post 4°C immersion in filtered chicken exudate

Immersion (days)	Regression coefficient (r) / sensitivity response (mV decade^{-1}) of biosensors[a]			
	1	2	3	4
1	**0.98 / 15.55**	-0.05 / -0.02	0.87 / 14.26	**0.99 / 49.35**
2	0.90 / 10.65	-0.09 / -0.06	0.86 / 12.97	[b]
3	0.68 / 5.36	-0.07 / -0.09	0.95 / 9.78	-0.46 / -69.73

[a] Sensors tested in quadruplicate. **Bold indicates passing performance;** minimum criteria for acceptable level of performance is r ≥0.98, sensitivity ≥10 mV decade^{-1}.
[b] Electrode was scratched; sensor could not be tested.

Twelve *Salmonella* biosensors were prepared and immersed in filter sterilized chicken exudate at a temperature of 4°C. At intervals of 24 hours for 3 days, 4 sensors were removed for testing in quadruplicate.

Regression analysis (Table 6) ascertained that only 2 of 12 biosensors met the minimum acceptable criteria of positive correlation and sensitivity. These two sensors were from four tested after immersion at 4°C for duration of one day. This gives a 50% success rate for the group of sensors immersed one day or less, which is in agreement with the longevity experiments conducted at 4°C for duration of one day as described previously. No other biosensors met the acceptable criteria. In order to determine if proper filtration had been performed, a standard loop of the filtered exudate had been cultured. This culture showed that there were still bacteria in the chicken exudate or contamination had occurred somewhere in the experiment. Only one type of colony was observed, but it was not identified. As well, a 10 μl aliquot of the filtered chicken exudate observed under darkfield microscopy revealed that there was an abundant amount of micrococci present; there were no rods seen. Therefore, sensor performance was not inhibited by the accumulation of *Salmonella*, but ultra filtration of the chicken exudate prior to sensor immersion did not improve the performance of the biosensor nor increase the working longevity of the biosensor.

b) Salmonella binding reversal.

Reversal of bacterial binding through oscillation was investigated. Eight *Salmonella* biosensors were prepared and tested pre- and post-rinsing with subphase; 4 with oscillation and 4 without oscillation. Regression analysis of pre-subphase and pre-

TABLE 7. Functional performance of biosensors pre- and post-oscillation at 200 mV decade^{-1}.

Sensor	Regression coefficient (r) / sensitivity response (mV decade^{-1}) of biosensors[a]		
	Preliminary testing	Post-oscillation testing[b]	Post-subphase testing[c]
1	**0.99 / 17.74**	**0.99 / 203.04**	
2	**0.99 / 37.85**	0.94 / 18.72	
3	0.90 / 26.75	0.97 / 23.34	
4	0.95 / 10.69	0.87 / 7.09	
5	**0.98 / 22.82**		**0.99 / 77.73**
6	**0.98 / 219.74**		[d]
7	0.93 / 18.92		0.86 / 6.79
8	**0.99 / 15.28**		-0.81 / -197.12

[a]**Bold indicates passing performance;** minimum criteria for acceptable level of performance is r $\geq$0.98, sensitivity $\geq$10 mV decade^{-1}.
[b] Oscillation step and cleaning with subphase.
[c] No oscillation step performed; cleaning with subphase only.
[d] Biosensor failed to plate; could not be tested.

oscillation testing ascertained that 5 of the 8 biosensors met the minimum acceptable criteria of positive correlation and sensitivity (Table 7). Post-subphase rinsing with no oscillation showed that only 1 of 4 biosensors performed at the acceptable level. As well, post-subphase rinsing with oscillation showed that only 1 of 4 biosensors performed at the acceptable level. None of the 8 biosensors tested post-subphase with or without oscillation performed at a level better than the pre-subphase rinsed biosensors. Therefore, oscillation through frequency of high energy does reverse binding of bacteria from the sensor surface.

c) Biosensors prepared using alternate antibodies. Twelve biosensors each were prepared using concentrated heat-treated and non heat-treated *Salmonella* antibody for a total of 24 biosensors. Isotherms were performed on both antibodies as described previously and the surface pressure for compression of the monolayer was determined to be 24 mN/m. The amount of antibody used to prepare monolayers was significantly less; only 25 µl as compared to 150 µl (of the commercial Denka-Seiken antibody) was required. This is due to the fact that the Auburn University derived antibody was much more concentrated than that of the commercially prepared antiserum.

Heat-treated antibody. Three of the 12 biosensors failed to reset to zero in what appeared to be a conductivity problem due to excessive amounts of scratches on the reverse side electrode of the crystal. Of the 9 biosensors that did function, regression analysis shows 5 met the minimum acceptable criteria of positive correlation and sensitivity. Heat treating the *Salmonella* antibody at 56°C for 30 minutes made the antibody more receptive to the *Salmonella* allowing for superior binding as opposed to non heat-treated anti-*Salmonella*. These results correlate well with those of Hseih (44).

TABLE 8. Functional performance of biosensors prepared using alternate antiserum

Sensor	Regression coefficient (r) / sensitivity response (mV decade^{-1}) of biosensors[a]	
	Heat-treated antiserum[b]	Non heat-treated antiserum
1	0.90 / 70.59	0.54 / 5.73
2	**0.98 / 39.25**	**0.98 / 21.33**
3	**0.99 / 39.25**	0.79 / 8.95
4	0.91 / 3.35	0.91 / 22.38
5	**0.99 / 27.02**	0.98 / 2.53
6	**0.98 / 35.11**	0.99 / 4.85
7	**0.99 / 11.58**	0.90 / 65.04
8	0.73 / 7.32	0.96 / 33.62
9	0.92 / 8.39	**0.99 / 24.13**

[a] **Bold indicates passing performance;** minimum criteria for acceptable level of performance is r $\geq$0.98, sensitivity $\geq$10 mV decade^{-1}.

[b] Heat-treated at 56°C for 30 minutes.

Non-heat treated antibody. Again, 3 of 12 biosensors failed to reset to zero due to what appears to be excessive amounts of scratches on the reverse side electrode of the crystal. Of the 9 biosensors that did function, regression analysis (Table 8) shows 2 met the minimum acceptable criteria of positive correlation and sensitivity for a success rate of 22%, which is far below the standard of 62% sensor reproducibility previously described. Although somewhat receptive, the non heat-treated antibody performed inferior to the heat-treated antibody. Therefore, heat-treated antibodies are better for sensor preparation, and should be used in order to achieve decent reproducibility.

d) Microscopic analysis of the biosensor. Visual characterization of the biosensor and monolayer was accomplished through SEM and darkfield microscopy.

Scanning electron microscopy. Micrographs show that the quartz crystal surface is quite jagged before the deposition of anti-*Salmonella* monolayers (Fig. 36). After deposition of monolayers (Fig. 37), the biosensor surface becomes more uniform in appearance, but the monolayer still shows an uneven appearance that agrees with the observation of Grunder et al. (37) and Rinia et al. (72). The adherence of *S. typhimurium* after voltage response testing (Figs. 38, 39) is evident and demonstrates antigen-antibody binding at the biosensor surface. These micrographs characterize the nature of the sensor and monolayer as called for by Ulman (92).

Darkfield microscopy. QC checkslides tested with *S. typhimurium* and viewed under darkfield microscopy showed positive agglutination responses as demonstrated by figure 35 and microscopically confirm SEM results seen in figure 39.

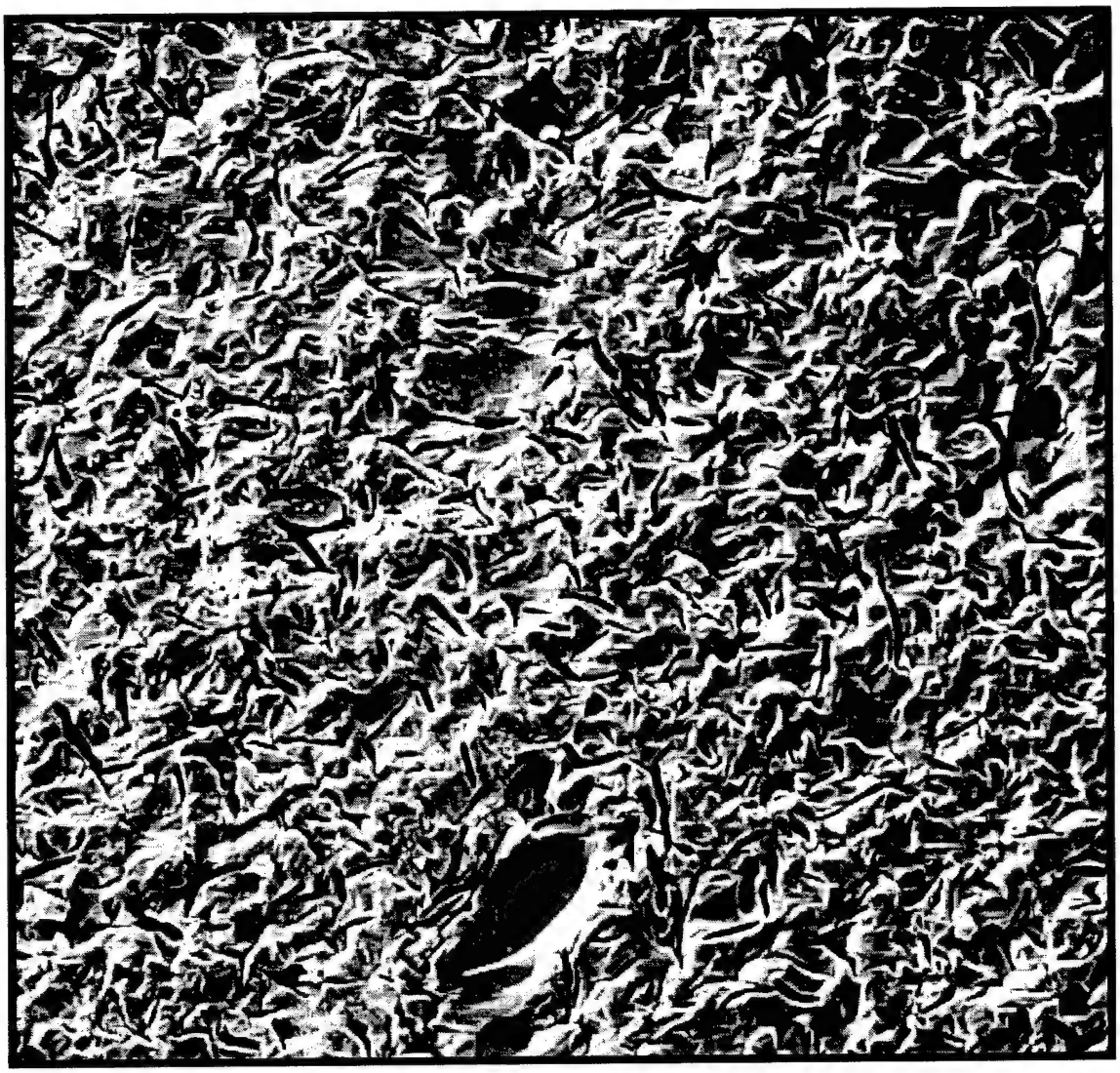

FIG. 36. Gold electrode surface of an unpolished quartz crystal sensor before deposition of *Salmonella* antibody monolayers. Scanning electron microscopy; magnification, ×1,000.

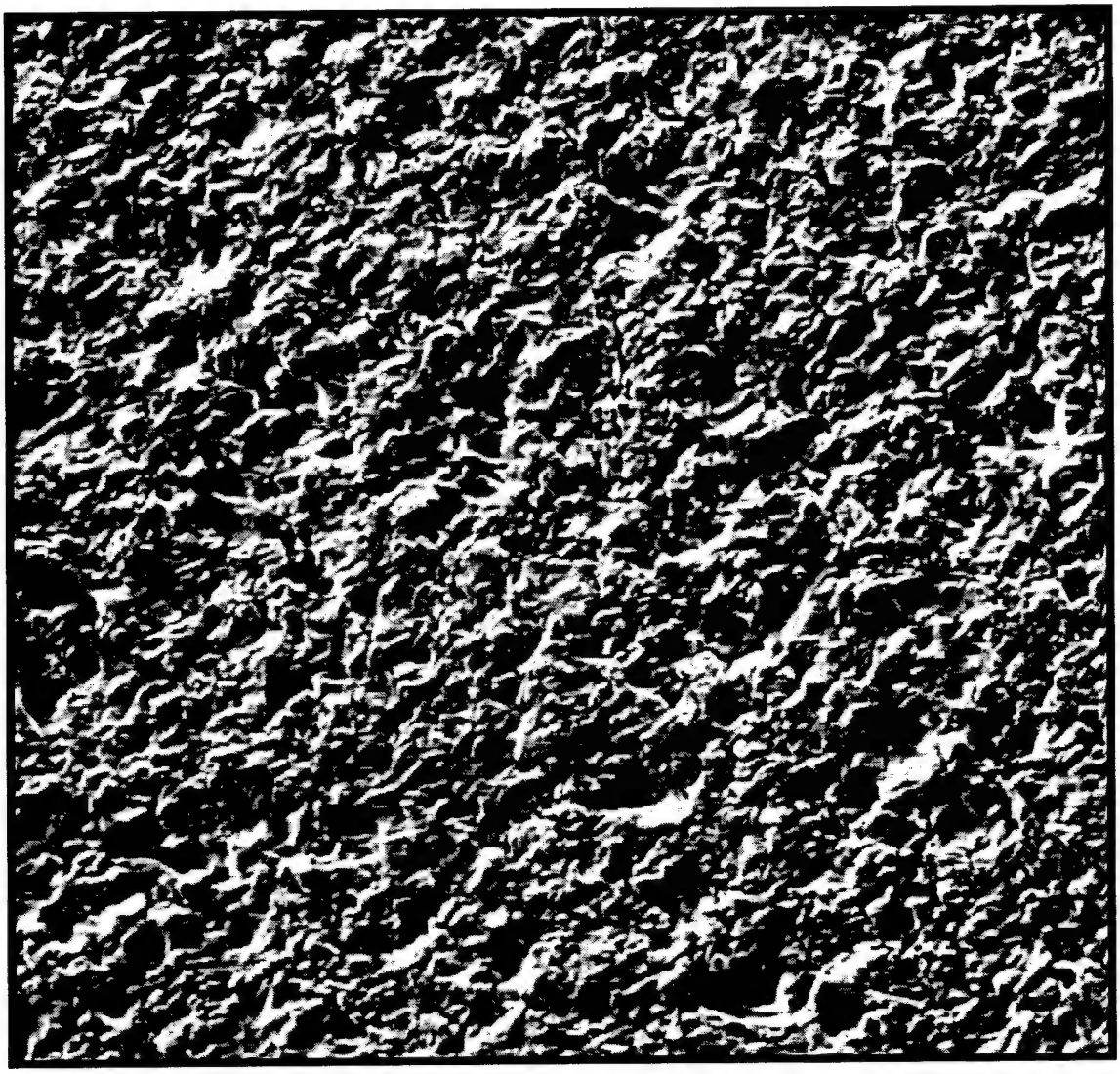

FIG. 37. Gold electrode surface of a quartz crystal sensor after the deposition of 37 *Salmonella* antibody monolayers. Scanning electron microscopy; magnification, ×5,000.

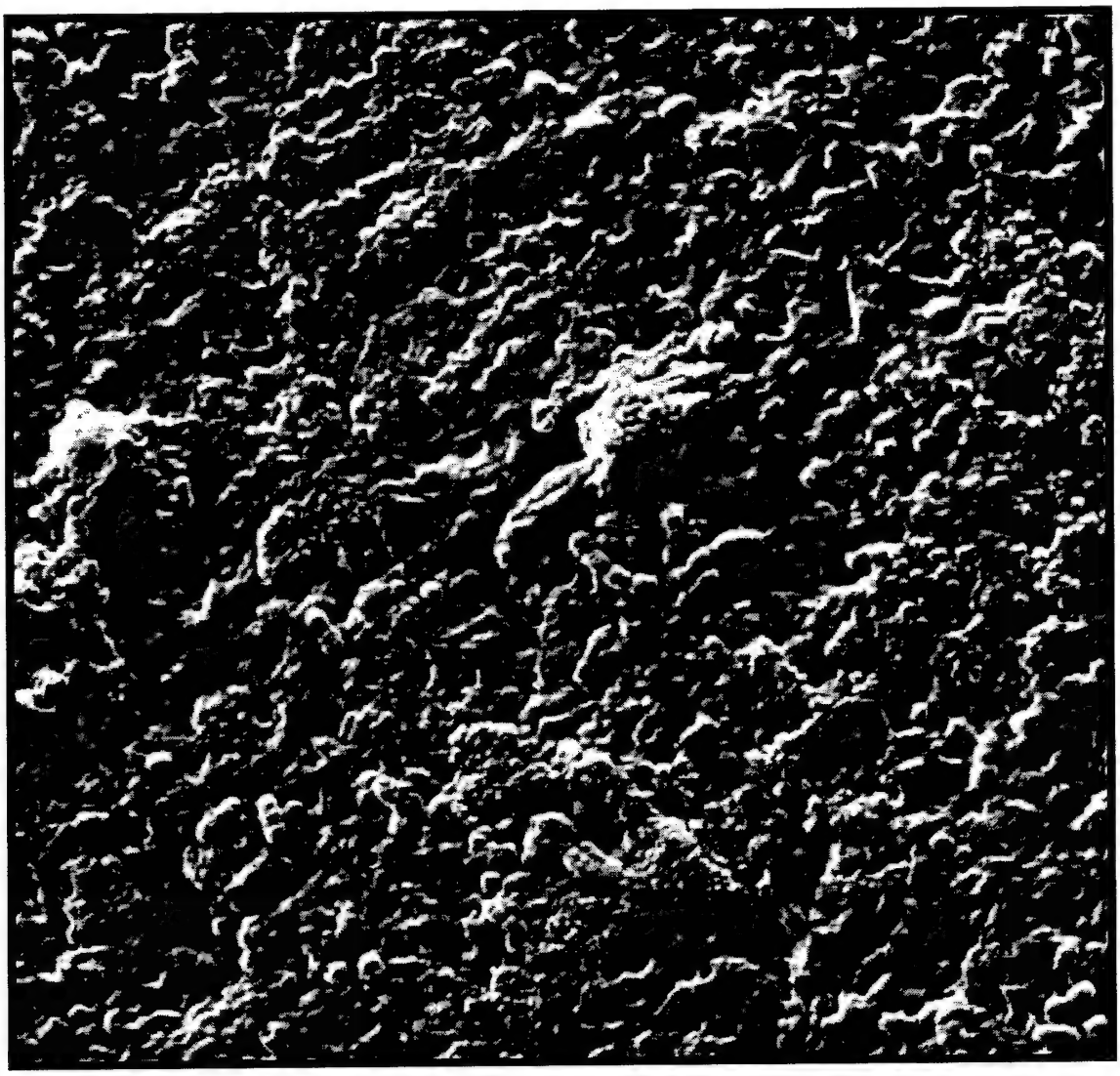

FIG. 38. Biosensor after testing with *Salmonella typhimurium* (arrow) exhibits binding via antibody interaction. Scanning electron microscopy; magnification, ×1,000, bar = 5μm.

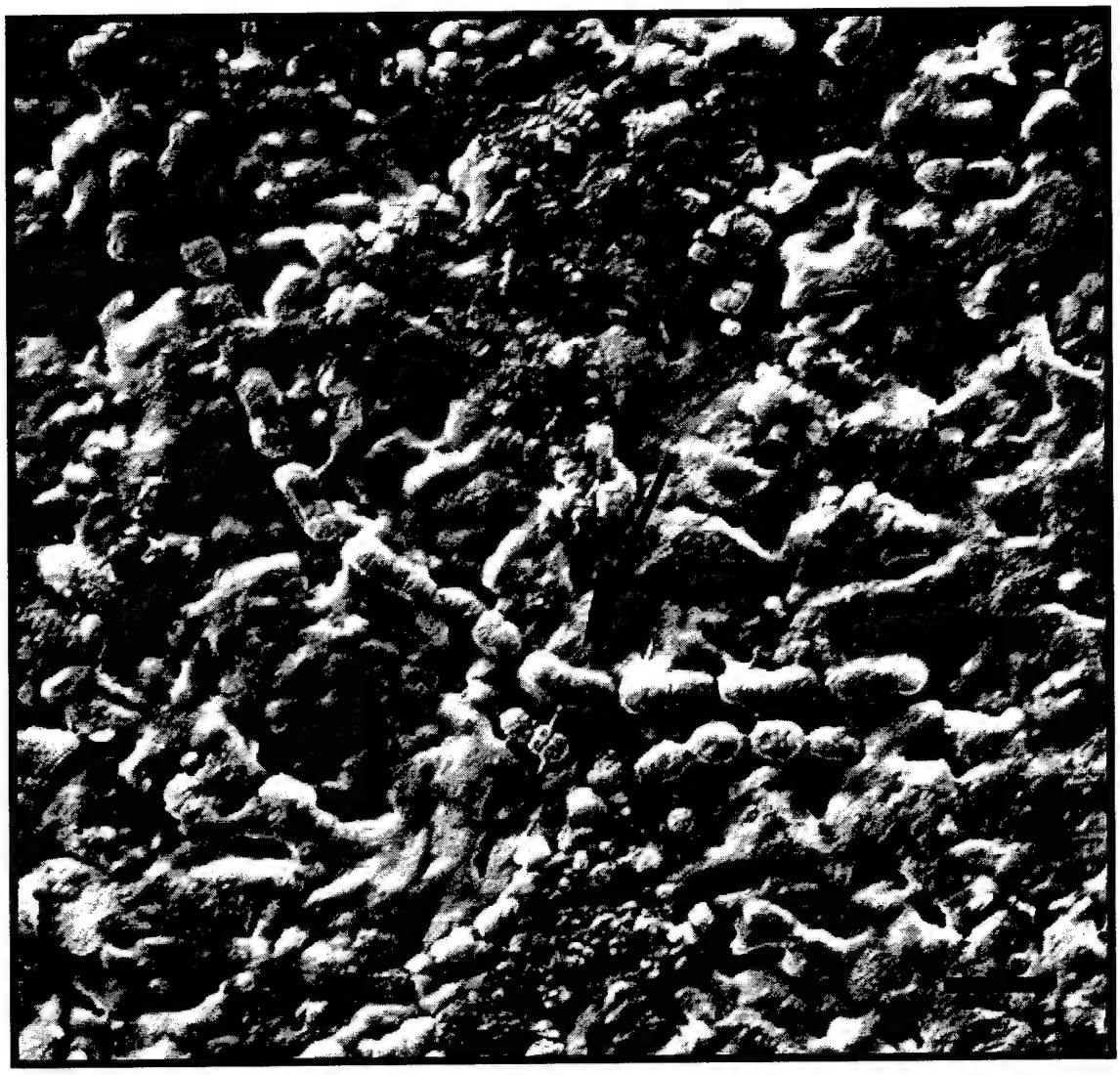

FIG. 39. Biosensor after testing with *Salmonella typhimurium* (arrow) exhibits binding via antibody interaction. Scanning electron microscopy; magnification, ×5,000, bar = 5μm.

CONCLUSIONS

This research was conducted to develop an improved biosensor capable of detecting *Salmonella* under immersed conditions within poultry packaging. With further refining, it could be an effective method for monitoring bacterial changes under any conditions that warrant detection of bacterial agents, including other food products and possibly biological warfare applications. The reproducibility of the sensor must be increased. Reusability of the sensor by reversing the binding of the bacteria must be investigated. The following conclusions were derived from the research conducted:

1. The functional duration of the *Salmonella* biosensor immersed in chicken exudate is between 32 and 48 hours at a temperature of 4°C. The sensors mean sensitivity is 25.8 mV decade^{-1} and has a detection range from ranges from 350 cells ± 150 cells to 10^{10} cells ml^{-1}. The detection ability of the biosensor degrades when immersed at high temperatures for any appreciable time. Microscopic observation of the monolayer through checkslides apparently shows bacterial buildup of indigenous bacteria on the monolayer with the duration of immersion. It is thought that this buildup may cause or contribute to the degradation of the sensors' detection ability.

2. The biosensor is highly specific for the detection of *Salmonella*. Other extraneous bacteria that may be present as normal flora in chicken exudate do not interfere with the detection of *Salmonella* when the biosensor is immersed in chicken exudate up to and between 32 and 48 hours at a temperature of 4°C.

3. Reversal of *Salmonella* binding was unaffected when the sensor was oscillated at a resonance frequency of higher energy. Ultra filtration of the chicken exudate prior to sensor immersion did not aid the performance of the biosensor nor increase the working longevity of the biosensor. The immunoreactant binding properties of the biosensor were greatest when *Salmonella* antibodies that had been heat-treated at 56°C for 30 minutes were used for preparation; sensors prepared using non heat-treated *Salmonella* antibodies did not function at a minimal acceptance level. *Salmonella* binding to antibody monolayers on the sensor was observed using scanning electron microscopy and darkfield microscopy.

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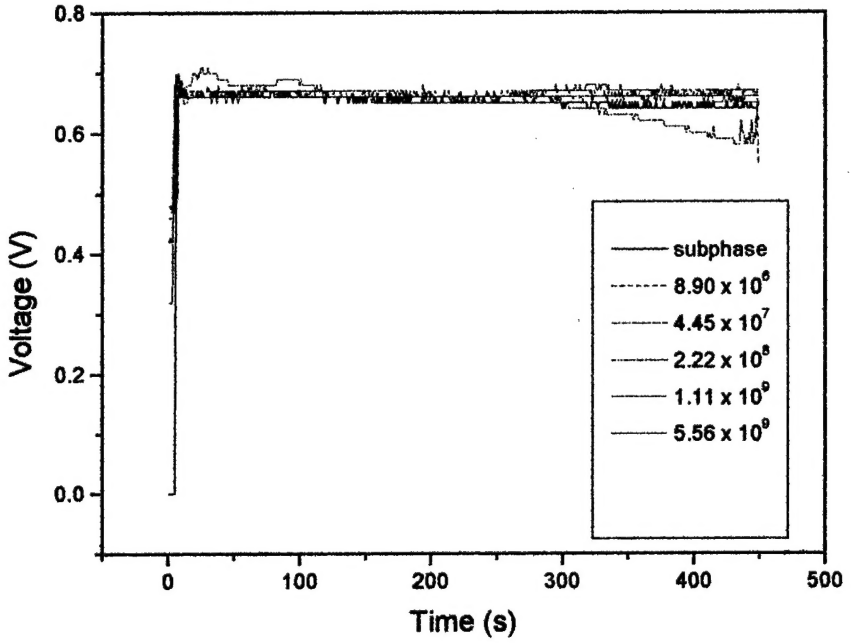
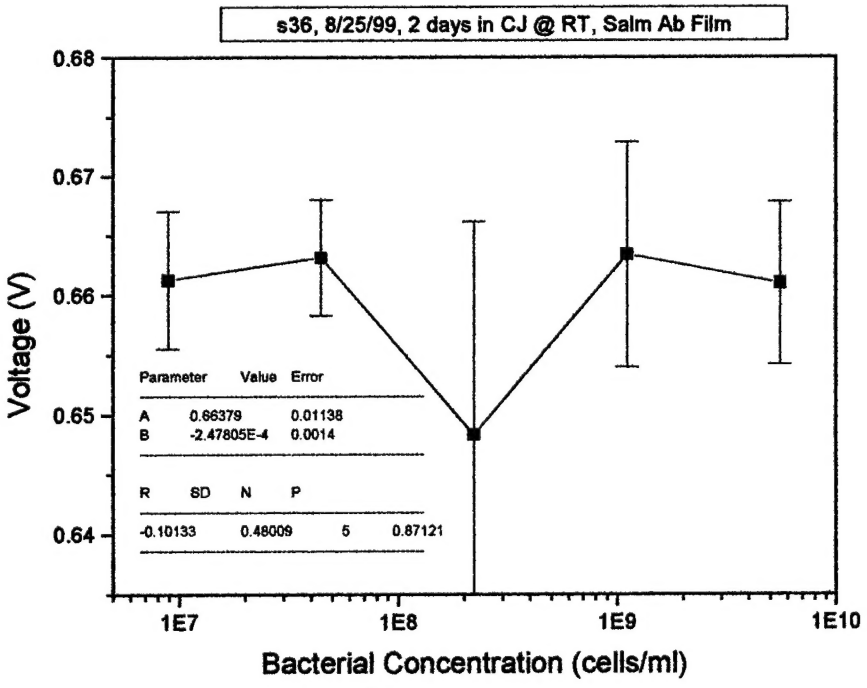
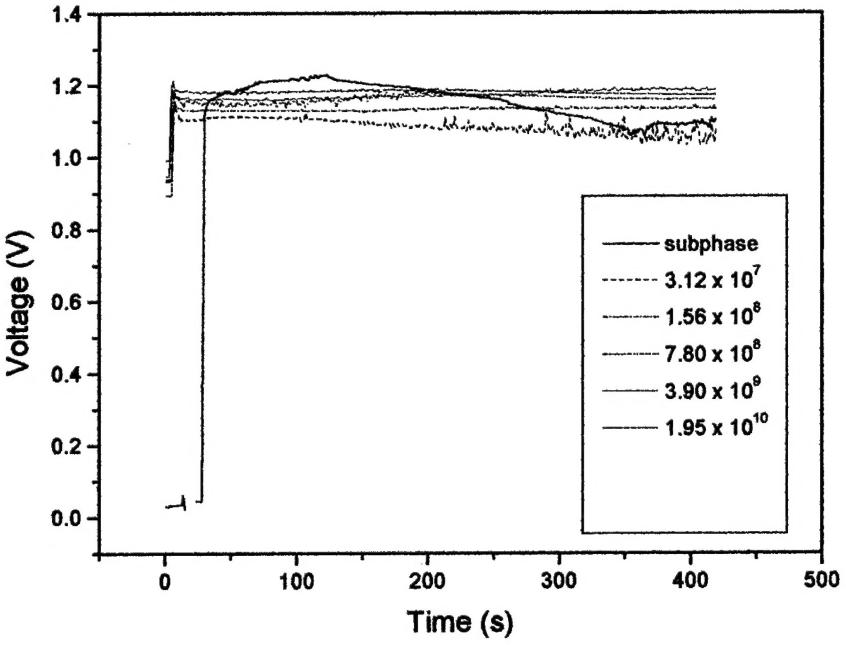
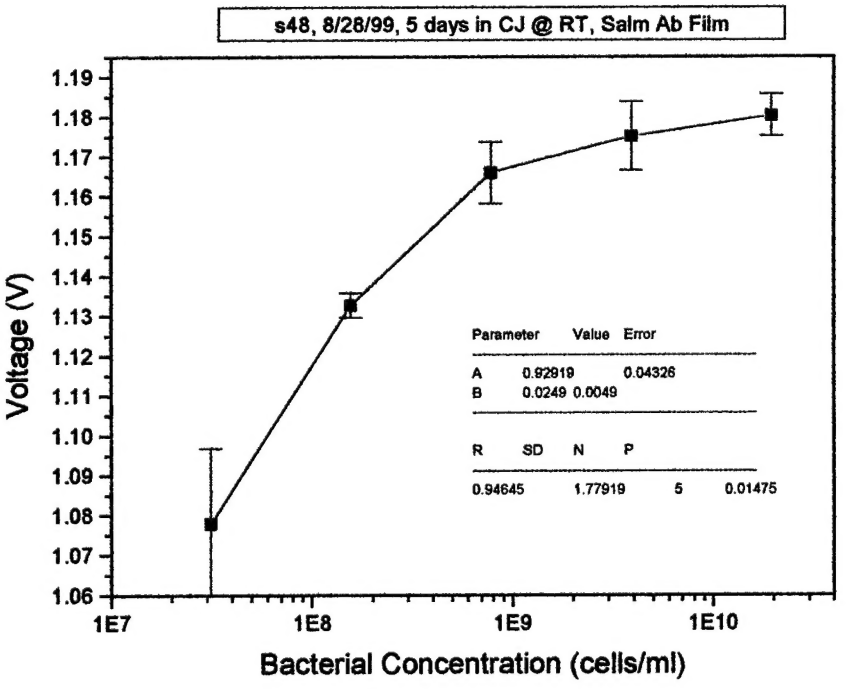
APPENDIX

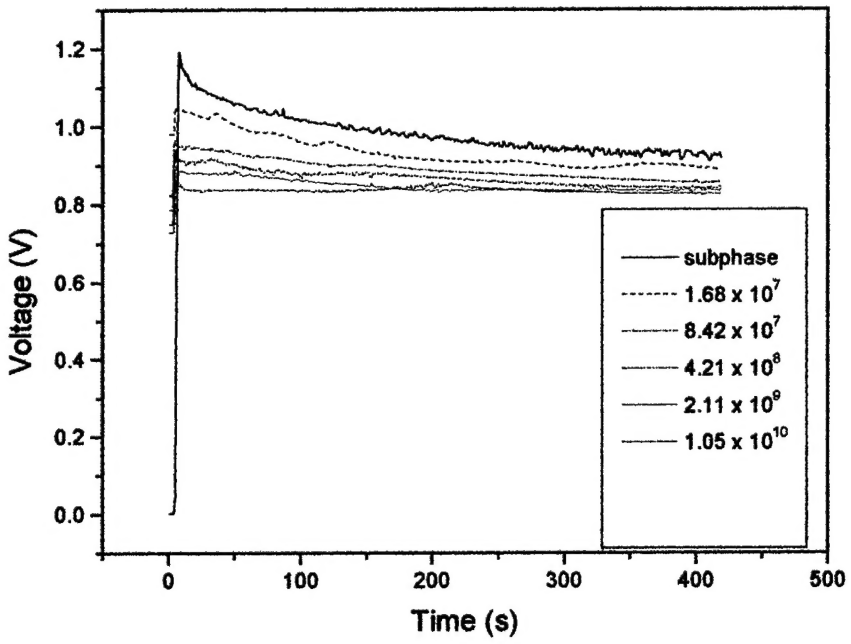
Three representative voltage response and dose response curves are shown for longevity experiments conducted at 23°C as denoted in figure 28 by biosensor numerical designations 8, 20, and 21. Numerical designation 8 (experimental data *Salmonella* sensor s36) represents immersion duration of 2 days in chicken exudate. Numerical designation 20 (experimental data *Salmonella* sensor s48) represents immersion duration of 5 days, and numerical designation 21 (experimental data *Salmonella* sensor s49) represents immersion duration of 6 days.

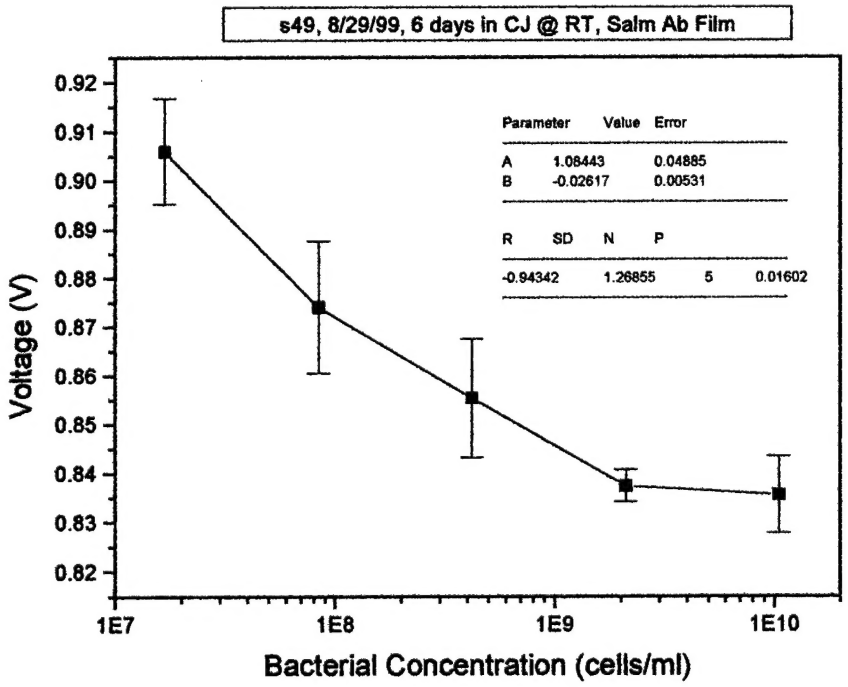

0.8
0.6
0.4
0.2
0.0
Voltage (V)
0
100
200
300
400
500
Time (s)
subphase
8.90 x 10^6
4.45 x 10^7
2.22 x 10^8
1.11 x 10^9
5.56 x 10^9


s36, 8/25/99, 2 days in CJ @ RT, Salm Ab Film
0.68
0.67
0.66
0.65
0.64
Voltage (V)
Parameter Value Error
A 0.66379 0.01138
B -2.47805E-4 0.0014
R SD N P
-0.10133 0.48009 5 0.87121
1E7
1E8
1E9
1E10
Bacterial Concentration (cells/ml)


Voltage (V)
Time (s)
subphase
3.12 x 10^7
1.56 x 10^8
7.80 x 10^8
3.90 x 10^9
1.95 x 10^10


s48, 8/28/99, 5 days in CJ @ RT, Salm Ab Film
Voltage (V)
Bacterial Concentration (cells/ml)
Parameter Value Error
A 0.92919 0.04326
B 0.0249 0.0049
R SD N P
0.94645 1.77919 5 0.01475


Voltage (V)
Time (s)
subphase
1.68 x 10^7
8.42 x 10^7
4.21 x 10^8
2.11 x 10^9
1.05 x 10^10


s49, 8/29/99, 6 days in CJ @ RT, Salm Ab Film
Parameter Value Error
A 1.08443 0.04885
B -0.02617 0.00531
R SD N P
-0.94342 1.26855 5 0.01602
Voltage (V)
Bacterial Concentration (cells/ml)

BARBAREE (MICB) **Form 9**

THE GRADUATE SCHOOL
Auburn University

REPORT OF MASTER'S CANDIDATE EXAMINING COMMITTEE

Committee Members:

James Barbaree ~~(Chair)~~ (Co-Chair) Thomas Pitta

Vitaly Vodyanoy (Co-Chair)

Bryan Chin

Date May 19, 2000

TO THE DEAN OF THE GRADUATE SCHOOL:

Eric V Olsen, candidate for the master's degree **(MS)**, having fulfilled all other qualifications, has this date been examined by us in the major field of Microbiology

and the minor field(s) of _____, and has defended his/~~her~~ thesis (title) Functional Durability of a Quartz Crystal Microbalance Sensor for the Rapid Detection of Salmonella in Liquids from Poultry Packaging

We hereby declare our

APPROVAL ~~DISAPPROVAL~~*

(Strike out the inappropriate term)

of the candidate for the award of the degree.

*In case of Disapproval, the Committee directs that the following disposition be made: _____

Signatures of Examining Committee:

James M. Barbaree (chair)

Vitaly Vodyanoy

Bryan Chin

I dissent from the above report: